EVALUATION OF ANTICANCER ACTIVITY OF VARIOUS EXTRACTS OF FRUIT OF *Amomum subulatum* Roxb. USING CERVICAL CANCER CELL LINE BY INVITRO METHODS

A dissertation submitted to

THE TAMILNADU Dr. M.G.R. MEDICAL UNIVERSITY
CHENNAI – 600 032

In partial fulfillment of the requirements for the award of degree of

MASTER OF PHARMACY
IN
PHARMACOLOGY

Submitted by

Reg. No: 261226051

INSTITUTE OF PHARMACOLOGY
MADRAS MEDICAL COLLEGE
CHENNAI – 600003

APRIL–2014

CERTIFICATE

This is to certify that the dissertation entitled "**EVALUATION OF ANTICANCER ACTIVITY OF VARIOUS EXTRACTS OF FRUIT OF *Amomum subulatum* ROXB. USING CERVICAL CANCER CELL LINE BY INVITRO METHODS".** Submitted by **Registration No. 261226051,** in partial fulfilment of the requirements for the award of the degree of **Master of Pharmacy in Pharmacology** by The Tamil Nadu Dr. M.G.R. Medical University, Chennai is a bonafide record of work carried out by her in the Institute of Pharmacology, Madras Medical College, Chennai during the academic year 2012-2014.

Dr.R.Vimala, M.D.,
Dean,
Madras Medical College,
Chennai-600003.

CERTIFICATE

This is to certify that the dissertation entitled "**EVALUATION OF ANTICANCER ACTIVITY OF VARIOUS EXTRACTS OF FRUIT OF *Amomum subulatum* ROXB. USING CERVICAL CANCER CELL LINE BY INVITRO METHODS".** Submitted by **Registration No. 261226051,** in partial fulfilment of the requirements for the award of the degree of **Master of Pharmacy in Pharmacology** by The Tamil Nadu Dr. M.G.R. Medical University, Chennai, is a bonafide record of work carried out by her in the Institute of Pharmacology, Madras Medical College, Chennai during the academic year 2012-2014.

Dr.R. Nandini, M.D.,
Director & Professor,
Institute of Pharmacology,
Madras Medical College,
Chennai -600003.

CERTIFICATE

This is to certify that the dissertation entitled "**EVALUATION OF ANTICANCER ACTIVITY OF VARIOUS EXTRACTS OF FRUIT OF *Amomum subulatum* ROXB. USING CERVICAL CANCER CELL LINE BY INVITRO METHODS".** Submitted by **Registration No. 261226051,** in partial fulfilment of the requirements for the award of the degree of **Master of Pharmacy in Pharmacology** by The Tamil Nadu Dr. M.G.R. Medical University, Chennai, is a bonafide record of work carried out by her under my guidance in Institute of Pharmacology, Madras Medical College, Chennai during the academic year 2012-2014.

Dr.K.M.Sudha, M.D.,
Associate professor,
Institute of Pharmacology,
Madras Medical College,
Chennai-600003.

ACKNOWLEDGEMENT

I humbly present this work to the ALMIGHTY GOD. Indeed my project is a small work done with the help of primitive persons at heart. So it is my bounded duty to promulgate them individually.

I whole heartedly express my high esteem and deep sense of gratitude to the respectable Dean **Dr.R.Vimala, M.D.,** Madras Medical College, Chennai-03, for providing me all facilities and support during the period of academic course work.

First of all, I sincerely express my deep sense of gratitude to **Dr. R. Nandini, M.D.,** Director and Professor, Institute of Pharmacology, Madras Medical College, Chennai for her guidance, encouragement and optimistic approach with praise for the successful completion of my project work.

I would like to express my heartfelt thanks and gratitude to my guide **Mrs. Dr.K.M.Sudha M.D.,** Associate professor, institute of Pharmacology, Madras Medical college for her Suggestions to complete my project. She is engrossed in this research at all stages and suggested numerous constructive observations to make this research successful.

I owe my special thanks to **Dr.B.Vasanthi,M.D.,**and **Dr.B.Kalaiselvi,M.D.,** Professors, Institute of Pharmacology, Madras Medical College for constant support and contribution my project work.

I owe my special thanks to **Dr. A.Suguna Bai, M.D.,** Associate Professor, Institute of Pharmacology, and Madras Medical College for constant support and contribution my project work.

My sincere thanks to **Dr.G. Chenthamarai, M.D., Dr.R.Malathi, M.D., Dr.Deepa , M.D.,** and **Dr. VijayaRani, M.D.,** Assistant professors, Institute of

Pharmacology, Madras Medical College, who had helped me in successful completion of my dissertation work.

I record my thanks to **Dr. A.C.Yegneshwaran,M.D.,** Tutor in clinical Pharmacology, institute of Pharmacology, Madras Medical college for his valuable suggestions to complete my project.

My Heartfelt thanks to **Mrs.R. Indumathi, M.Pharm.,** and **Mrs .M.Sakthi Abirami, M.Pharm.,** Tutors, Institute of Pharmacology, Madras Medical College for their constant support and encouragement throughout my project.

A special word of thanks goes to **the non-teaching staff members** and **Lab Technicians** of Institute of Pharmacology, Madras Medical College, Chennai-03.

My sincere thanks to my dear friend **Ms.N.Ramya** for her constant support and encouragement throughout my project.

I am very thankful to my senior **Mr. D. Sundara vadivel, Mr. P. Gopinath** for their help and support during the study.

My special thanks to**, Ms.K.Abirami, Mrs.Devi, Mrs.G.Geethapriya, Mr.R.V.Sivasubramani, Mr.M.Pasupathiraja, Mr.J.Sivaraman, Mr.C.Vijay kumar** College of Pharmacy, Madras Medical College, for their encouragement and support.

I am grateful to my parents and family **Mr. Lal chand yadav and Mrs. Siva kumari yadav, Aryan** for their blessings upon me to make this accomplishment possible.

THANKS TO ONE AND ALL

CONTENTS

Dedicated to My Family

1. INTRODUCTION

According to the World Health Organization, cancer is the second leading cause of death in developed countries and is among the three leading causes of death for adults in developing countries (WHO). A steadily increasing proportion of elderly people in the world will result in approximately 16 million new cases of cancer by the year 2020 (IARC).[1]

Due to environmental conditions and risk factors such as age, smoking, alcoholism, obesity, poor diet, lack of exercise, prolonged exposure to sunlight (UV Radiations) there is risk of developing cancer in every individual.[2]

Cervical cancer is the 3rd most common cancer in women worldwide and the most common cancer causing death in developing countries.[3] Sexually transmitted HPV infection is the most important risk factor for cervical intraepithelial neoplasm and invasive cervical cancer.

In India with a population of approximately 365.71million women above 15 years of age are at risk of developing cervical cancer. Currently approximately 132,000 new cases diagnosed and 74,000 death annually in India, accounting to nearly 1/3 of global cervical cancer deaths. Indian women face a 2.5% cumulative life time risk and 1.4% cumulative death risk from cervical cancer.[4]

Till date there is no exact treatment for cancer. Mostly cancer gets diagnosed only at the end stage, where treatment mostly becomes resistant. Treatment includes surgery, chemotherapy, and radiotherapy. Chemotherapy not only kills the cancerous cells but also the normal cells and makes the patients more vulnerable. So, traditional medicines can be used to treat the patients with less or no side effects as with allopathy.

HERBAL medicine is still the mainstay of about 75–80% of the world population, mainly in the developing countries, for primary health care because of better cultural acceptability, better compatibility with the human body and lesser side effects. Today approximately 25% of all prescription drugs are derived from trees, shrubs or herbs.[5]

Amomum subulatum is traditionally used as an appetizer. Its phytochemical studies have revealed that it contains flavonoids, terpenoids and it has anti-oxidant properties which are important for anticancer activity.[39]

A HeLA cell is a cell type in an immortal cell line used in scientific research. It is the oldest and most commonly used cell line. The cell line was derived from cervical cancer. HeLa cells, like other cell lines, are termed immortal in that they can divide an unlimited number of times in a laboratory cell culture plate as long as fundamental cell survival conditions are met (*i.e.,* being maintained and sustained in a suitable environment).[6]

In *Amomum subulatum* antioxidant activity[39] has been proved, so present study was undertaken to investigate invitro anticancer activity of various extracts of *Amomum subulatum* using HeLA cell line.

2. OBJECTIVE

- To identify the Phytochemical constituents of various extracts of fruits of *Amomum subulatum*
- To evaluate the cytotoxic activity of ethanolic extract of fruits of *Amomum subulatum* in cervical cancer cell line HeLa and to establish IC50 concentration.
- To evaluate the Apoptotic effect of ethanolic extract of fruits of *Amomum subulatum*.
- To study the Gene expression levels in *Amomum subulatum* treated HeLa cancerous cell line.

3. REVIEW OF LITERATURE

3.1 CANCER:

Cancer is a condition where cells in a specific part of the body grow and reproduce uncontrollably. The cancerous cells can invade and destroy surrounding healthy tissue, including organs.

Medical Term: Malignant Neoplasm.[7]

It is a class of disease in which a group of cells display the characteristics of

- ✓ **Uncontrollable Growth** [Growth and division beyond normal limits]
- ✓ **Invasion** [Intrusion on and destruction of adjacent tissues]
- ✓ **Metastasis** [Spread to other locations in the body via lymph or blood]

Cancer sometimes begins in one part of the body and spreads to other areas, called as metastasis.[8]

3.2 CANCER CLASSIFICATION:

There are four main cancer classification namely,

- ✓ **Carcinoma :** The abnormal cells start their growth in the epithelial tissues. Eg . cancers of lungs, prostate, colon, pancreas, breast, cervical cancer.
- ✓ **Sarcoma :** Tumour grows in soft connective or supportive tissues. Eg . cancers of bone, muscles, nerves, fibrous tissues, blood vessels.
- ✓ **Lymphoma :** Tumour that develops in the lymphocytes of immune system, usually in lymph nodes. Eg . non-Hodgkin's lymphoma.
- ✓ **Leukemia:** The location of cancerous cells is the bone marrow. It affects blood cells. Eg . ALL, CLL, AML, CML.[9]

3.3 CAUSES:

Cancers are primarily caused due to environmental factors (90-95%) and due to genetic factors(5-10%). Common environmental factors that contribute to cancer include

Tobacco (25-30%)

Diet and Obesity (30-35%)

Infections (15-20%)

Radiation (up to 10%)

Stress, lack of physical activity

Environmental pollutants.[10]

Cancer pathogenesis is linked to **DNA mutation** that impact cell growth and lead to metastasis. Substances causing DNA mutations are mutagens and mutagens that cause cancers are known as **Carcinogens**. There exist a strong link between tobacco use and cancer in the lung, larynx, head, neck, stomach, bladder, kidney, oesophagus and pancreas. Tobacco smoke contains nearly **fifty known carcinogens,** responsible for about one in three of all cancer deaths in developed world.

Diet, physical activity and obesity contribute to 30-35% of cancer death. Physical inactivity contributes to cancer risk by having negative effects on immune system and endocrine system apart from body weight. Diets low in vegetables, fruits and whole grains, high in processed or red meat are linked with number of cancers. Eg. Betel nut chewing with oral cancer, high salt diet linked to gastric cancer.[11]

Infectious diseases contribute to 18% of cancer death worldwide. Viruses are usual infectious agents, but bacteria and parasite too have their contribution. Viruses that cause cancer are termed as **Oncovirus.**

Eg. Human papilloma virus [Cervical carcinoma], Hepatitis B and C virus [Hepatocellular carcinoma

Invasive cancers of about 10% are related to **radiation** exposure including both ionizing and non-ionizing radiation.

3 to 10% of cancers are due **to inherited genetic defect**. Eg . breast cancer and ovarian cancer(75%) is due to inherited mutations in genes **BRCA1 and BRCA2.**[10,11]

3.4 INCIDENCE AND MORTALITY OF CANCER:[12]

Cancer is a leading cause of death worldwide and accounting for 7.6 million deaths in 2008.

The main types of cancer are:

- ✓ Lung(1.37 million deaths)
- ✓ Stomach(736000 deaths)
- ✓ Liver(695000 deaths)
- ✓ Colorectal(608000 deaths)
- ✓ Breast(458000 deaths)
- ✓ Cervical cancer(2,88,000 deaths)

70% of all cancer deaths occurred in low and middle income countries. **Cervical cancer is the 3rd most common cancer in women.**

3.5 CERVICAL CANCER

DEFINTION:

Cervical cancer is a malignant neoplasm arising from cells originating in the cervix uteri. Cervix is the lower, narrow portion of the uterus where it joins with the top end of the vagina.

3.6 TYPES OF CERVICAL CANCER:[13]

- Squamous cell cancer
- Adenocarcinoma

Squamous cells are the flat skin like cells that cover the outer surface of the cervix. Squamous cell cancer is most common type.

Adenomatous cells are gland cells that produce mucus. The cervix has these gland cells scattered along the inside of the passageway that runs from the cervix to the womb.

3.7 CAUSES OF CERVICAL CANCER:[14]

HUMAN PAPILLOMAVIRUS:

Human papillomavirus type 16 and 18 are the cause of 75% of cervical cancer globally. Woman who have many sexual partners have greater risk.

According to American cancer society and other organizations the patient must have been infected with human papillomavirus to develop cervical cancer and is hence a sexually transmitted disease.

CIN is usually the result of an infection from the human papilloma virus (HPV). HPV is a very common virus that can affect the cells of the cervix. It's mainly passed on during sex.

Having sex at an early age and having several sexual partners can increase the risk of catching HPV and developing cervical cancer.

But many women who have only had one sexual partner have HPV at some point in their life, and may go on to develop CIN or cervical cancer.

SMOKING:

Smoking has also been linked to the development of cervical cancer. Female smoker has a higher chance of CIN3 (cervical intraepithelial neoplasia) is a potentially premalignant transformation and abnormal growth of squamous cells on the surface of the cervix lead to cancer most of them have assistance of human papillomavirus.

A WEAKENED IMMUNE SYSTEM

Having a weakened immune system may allow CIN to develop into cancer. The immune system can be weakened by smoking, a poor diet and infections such as HIV/AIDS.

3.8 PATHOPTHYSIOLOGY:

Cancer is generally a disease attributed to failure of regulation of tissue growth. For a normal cell to transform into cancerous cell, the genes which regulate the cell growth and differentiation must be altered. **Oncogenes** and **Tumour suppressor genes** are two types of cancer genes. **Oncogenes are genes that positively influence tumour formation, whereas tumour suppressor genes are those that negatively impact tumour growth.** Both oncogenes and tumour suppressor genes exert their effect on

tumour growth through their ability to control cell division (cell birth) or cell death (apoptosis). Normal function of tumour suppressor gene is usually to restrain cell growth and this function is lost in cancer.

Genetic changes can occur at different levels and by different mechanisms. Gain or loss of an entire chromosome can occur through errors in mitosis. More common are mutations which are changes in the nucleotide sequence of genomic DNA. Large scale mutations involve the deletions and insertions which affects the expression of the gene. Mutation in the error correcting machinery of a cell might cause that cell to accumulate errors more rapidly. Mutation in oncogenes might cause the cell to reproduce more rapidly and more frequently than normal cells. Mutation causes loss of tumour suppressor genes, which disrupts apoptosis signalling pathway and makes the cell immortal.[15]

p53

p53 is known by names like **'tumour protein 53'** or 'protein 53'. It is a tumor suppressor protein present in humans and it is encoded by gene TP 53 which is located in the 17th chromosome. It plays a significant role in regulation of cell cycle and helps in preventing cancer. It plays a major role in preventing the mutation of genome and has been rightly described as **'guardian of genome'.**

p53 gene is not active in cells were DNA has not been damaged. In case of DNA damage, it suspends the cell cycle until the damage has been repaired. If there is a mutation in p53, the cell cycle continues without any restriction and also reproduces the damaged DNA leading to uncontrolled cell proliferation finally ending up in Cancer.

Apoptosis induced by p53 involves the role of many mediators, important one being bax protein. Bax protein is a member of Bcl-2 protein family. p53 binding site located in the regulatory region of the gene which directly activates transcription of Bax gene and when over expressed Bax induces apoptosis.[16]

Bcl-2:

When the homeostatic balance between the cell growth and cell death is disturbed, it leads to development of cancer. Apoptosis is the process of programmed cell death, when altered gives rise to a number of human diseases like **cancer**, autoimmune disorders and some viral infections. **Bcl-2 and its family of proteins play a significant role in regulation of Apoptosis.**

Bcl-2 family includes both pro and anti-apoptotic proteins. Anti-apoptotic proteins of this family includes Bcl-2, Bcl-xL, Mcl-1, Bcl-w and A-1 which suppresses the cell death, whereas pro-apoptotic proteins of this family includes Bax, Bak, Bik, Bad and Bid which causes promotion of cell death.

Bax gene was the first pro-apoptotic member of the Bcl-2 family, which promotes apoptosis by competing with Bcl-2. Tumour suppressor protein p53 up regulates the expression of Bax and Bax is proven to be involved in apoptosis mediated by p53. Majority of Bax is found in cytosol and upon initiation of apoptosis; it undergoes a conformation shift and fits into outer mitochondrial membrane. Further it results in the release of cytochrome c and other pro-apoptotic factors from mitochondria leading to apoptosis.[17]

P21:

p21 is a potent cyclin-dependent kinase inhibitor (CKI). The p21 protein binds to and inhibits the activity of cyclin-CDK2, -CDK1, and -CDK4/6 complexes, and thus functions as a regulator of cell cycle progression at G_1 and S phase. In addition to growth arrest, p21 can mediate cellular senescence. The expression of this gene is tightly controlled by the tumor suppressor protein p53, through which this protein mediates the p53-dependent cell cycle G_1 phase arrest in response to a variety of stress stimuli. Growth arrest by p21 can promote cellular differentiation. p21 therefore prevents cell proliferation.

Rb PROTEIN:

The Rb protein plays a key role in regulating the cell cycle. It is expressed in every cell type, where it exists in an active hypophosphorylated and in active hyperphosphorylated state. In its active state, Rb serves as a brake on the advancement of cells from the G1 to the S-phase of the cell cycle. If Rb is lost or made nonfunctional through mutation, this brake on the cell cycle is released and cells move into S-phase unrestrained. Specifically, Rb normally binds to and inactivates the E2F transcription factor. Loss of Rb results in activation of E2F. E2F binds the promoter of the cyclin E gene and in turn causes increased expression of the cyclin E gene and synthesis of Cdk2-cyclin E complexes, which then drives the cell cycle.[18,19]

In cervical cancer E6 and E7 are human papillomavirus proteins which enhances P53 degradation causing a block in apoptosis and decreased activity of the P21 cell cycle inhibitor. E7 associates with P21 and prevents its inhibition of cyclin-CDK4 complex. E7 can bind to RB proteins removing cell cycle restriction. The net effect of E6 and E7 proteins is to block apoptosis and remove the restraints to cell proliferation.[20,21]

3.9 SYMPTOMS OF CERVICAL CANCER

Very early-stage cervical cancer may have no symptoms. This means it is important to attend regular cervical screening, so that any cell changes can be picked up early.

The most common symptom of cervical cancer is abnormal vaginal bleeding, usually between periods or after sex.

Postmenopausal may find they have some new bleeding.

Symptoms of cervical cancer can also include a smelly vaginal discharge and discomfort during sex.[22]

3.10 STAGING AND TREATMENT OF CERVICAL CANCER:[23,24,25,26,27]

STAGE 0 CANCER:

Carcinoma in situ is treated with local ablative or excisional measures such as cryosurgery, laser ablation, and loop excision. Surgical removal is also preferred. After treatment these patients require lifelong surveillance.

STAGE I - A1 CANCER:

The treatment choice for this stage is surgery. Total hysterectomy, radical hysterectomy and conisation are accepted procedure.

According to National comprehensive cancer network (NCCN) guidelines, pelvic radiation therapy is currently a category 1 recommendation for women with stage IA disease and negative lymph nodes after surgery who have high-risk factors (eg, a large primary tumour or lymphovascular space invasion)

STAGE I - A2, IB, OR IIA CANCER:

Patients with stage IB or IIA disease, there are 2 treatment options

- **Combined external beam radiation with brachytherapy.**
- **Radical hysterectomy with bilateral pelvic lymphadenectomy.**

Radical vaginal cervicectomy, with pelvic lymph node dissection is appropriate for fertility preservation in women with stage IA2 disease and those with stage IB1 disease whose lesions are 2cm or smaller.

For patients with IB2 or IIA cancer and tumours larger than 4cm, radiations and chemotherapy is selected in most cases.

STAGE IIB, III OR IVA CANCER:

For locally advanced cervical carcinoma (stage IIB, III and IVA), radiation therapy was the treatment of choice for many years.

The use of cisplatin – based chemotherapy in combination with radiation has become the standard of care for primary management of patients with locally advanced cervical cancer.

STAGE IVB AND RECURRENT CANCER:

Radiation therapy is used alone for control of bleeding and pain, whereas systemic chemotherapy is used for disseminated disease. For disease recurring after chemotherapy and radiation therapy, a disease – free interval of more than 16 months is considered to designate the tumour as platinum sensitive. The standard care is chemotherapy with a paclitaxel and cisplatin.

3.11 PLANT PROFILE

Plant name : *Amomum subulatum*

Family : zingiberaceae

Genus : Amomum

Vernacular names :

English : Great Indian cardamom

Hindi : Bari iliachi

Tamil : Katu elam

Telugu : Nalla elakulu

Malayalam : Kattelam

Habitat : Mountainous areas

Habit : Perennial bush

Part used : Fruit

DESCRIPTION:

It is a perennial bush of the ginger family, with sheathed stems reaching 10-12 feet in height. It has a large tuberous rhizome and long, dark green leaves 30-60 cm (1-2 ft) long, 5-15 cm (2-6") wide. Trailing leafy stalks grow from the plant base at ground level, these bear the seed pods. The flowers are white with blue stripes and yellow borders. The fruit is a small capsule with 8 to 16 brown seeds; the seeds are used as a spice.[28]

CHEMICAL CONSTITUENTS:

Chemical constitutes present are flavonoids, carbohydrates, amino acids, triterpenoids, glycosides, tannins, 1,8 cineole, limonene, terpinenes, pinenes, gallic acid, protocatechuic acid.[28]

USES:

The most common use of Larger or Greater Cardamom is as a mouth freshener.

- ✓ It is used in the cure of dyspnoea, cough, itching, abdominal pain.
- ✓ Its seeds are credited with stimulant, stomachic and astringent properties.
- ✓ It is used to treat and prevent sore throats.
- ✓ It is also prescribed in the treatment of indigestion and nausea.
- ✓ It is used as antispasmodic.

The essential oil, obtained from the steam distillation of the crushed seeds of Larger Cardamom, contain cineole. Cineole gives the herb its aroma as well as its digestive and appetizer properties.

Its antioxidant properties have been reported in the *Journal of the Association of Physicians* in India.[28]

Figure-1: Plant of *Amomum subulatum*

Figure-2: Seeds of *Amomum subulatum*

3.12 PHARMACOLOGICAL ACTIVITY IN *Amomum subulatum*:[28,29,30,31]

Analgesic activity;

Shukla et al.,(2010) reported Methanolic extract of seeds of *A. subulatum* at dose 100 and 300 mg/kg and ethyl extract at dose of 200 and 400 mg/kg, showed significant ($p<0.001$) analgesic effect.

Anti-inflammatory:

Alam et al,.(2011) reported the ethanolic and aqueous extract showed anti-inflammatory activity with a dose of 100mg/ml and 200mg/ml respectively .

Anti-microbial activity:

Kumar et al,.(2010) reported *A. subulatum* has a wide variety of secondary metabolites such as tannins, alkaloids and flavonoids having antimicrobial activities. Petroleum ether extracts of large cardamom showed antimicrobial activity and were found active on *Staphylococcus aureus*, *Escherichia coli* (-ve) and *Pseudomonas aeruginosa* (-ve) bacteria.

Aneja and joshi.,(2009) reported the acetonic, methanolic and ethanolic extracts of *A.subulatum* showed antimicrobial inhibitory activity against two bacteria causing dental caries, *Streptococcus mutans* and *S. aureus* and two fungi *Candida albicans* and *Saccharomyces cerevisiae.*

Jain and agarwal.,(1976) reported the essential oil from seed was found to have significant inhibitory effect against various keratinophilic and dermophytic fungi.

Antioxidant activity:

Verma et al.,(2010) and kikuzaki et al.,(2001) Antioxidant is the term used to describe a dietary component that can function to decrease tissue damage by reactive oxygen. Antioxidants thus have great value in preventing the oxidative diseases such as chronic fatigue, premature ageing symptoms, degenerative cardiovascular and neurovascular diseases associated with ageing . The 1,8-ceineoil and alphaterpineol, protocatechualdehyde and protocatechuic acid present in the seeds of *A. subulatum* showed antioxidant activity and has potential health benefits by inhibiting lipid peroxidation . The seed has antioxidant activity on hepatic and cardiac antioxidant enzymes and is attributed to their ability to activate antioxidant enzymes.

Antiulcer activity:

Jafari et al.,(2001) reported Crude methanolic extract of the fruits of *A.subulatum* shows antiulcer activity.

Sen et al.,(2009) reported methanolic fraction, petrol soluble fraction, ethyl acetate soluble fraction, ethyl acetate soluble fraction produces significant ulcer protection against ethanol induced ulcer.

Farah et al.,(2009) reported essential oil of *A. subulatum* inhibit ulcer formation by 60.91% ($p<0.001$), significantly in ethanol and aspirin induced gastric ulcer.

Cardio-adaptogen activity:

Verma et al.,(2010) reported *A. subulatum* has protective effect against the effect of acute or severe stress induced myocardial damages . Regular consumption of greater cardamom may therefore be useful in treatment for patients with Ischemic Heart Disease (IHD), facing regular stressful conditions.

Hypolipidaemic activity:

Hafidh et al.,(2009) reported that large cardamom has significant ability to suppress lipid peroxidation due to the presence of polyphenol content. *A. subulatum* supplementation increases the antioxidant enzyme activities, and the lipid conjugated dienes and hydroperoxides.

Yadav and bhatnagar (2007) reported the inhibition of lipid peroxidation in rat liver homogenate due to their polyphenol content, strong reducing power and superoxide radical scavenging activity.

Acute toxicity studies:

Acute toxicity study was conducted for methanolic extract of *A.subulatum* seeds by stair case method following OECD guidelines-425 , 2001. There was no lethality observed up to a dose of 3000mg/kg.

Hepatoprotective activity:

Mihir parmar, purvi shah, vaishali Thakkar, Tejal R. Gandhi.,(2009) The hepatoprotective activity of methanolic extract of *A. subulatum* against ethanol induced hepatotoxicity in albino wistar rats. Hepatoprotective activity o of methanolic extract of *A. subulatum* were evaluated by estimation of SGOT, SGPT, ALP, TP, total bilirubin and direct bilirubin in treated rats. *A. subulatum* seeds significantly reduced SGPT, SGOT, ALP and increased TP levels indicating their hepatoprotective activity.

Anti-diabetic activity:

Patel Amit et al.,(2012) Anti-diabetic activity of *Amomum subulatum* Seed was evaluated in fructose fed metabolic syndrome in rat. Oral administration of acetone and methanol extract of *A. subulatum* extracts revealed a significant increment of serum

insulin levels, higher reduction in hyperglycemia when compared to the diabetic control rats . The histological studies of the endocrine region of pancreas of diabetic animals revealed that shrinkage of β cells of islets of langerhans. Animals treated with both extracts of *A. subulatum*, revealed restoration of β-cells. This activity of acetone and methanolic extract might be due to presence of phenolics like protocatechuic acid.

3.13 HeLa cell line:

The HeLa cell line is an immortal cancer cell line. It is termed "immortal" because the cells can replicate indefinitely in culture. The original cells came from a visible malignant cervical tumor in the body of a 31-year-old African American woman named Henrietta Lacks. The cells were taken early in 1951 and given to Dr. George Gey, who produced the original cell line. Henrietta Lacks died the same year, in October, from the very cancer cells that have since been used to save thousands and thousands of lives.

Now there are many strains of HeLa cells all over the world that have evolved in cell culture, and it is estimated that more HeLa cells have been cultured than were in Henrietta Lacks body to begin with.[32]

MTT ASSAY:

Sheng-hua zhang et al.,(2007) soyasaponins are present in legumes and soyabeans are the primary dietary source of saponins. SS-II the second fraction of soyasaponins, was separated by column chromatographic method with D101 A macroporous resin from soyabean. Study at the concentration range of 100-400 mg/L, SS-II had obvious cytotoxic effect on Hela cells by MTT assay.[33]

Aied M Alabsi et al.,(2012) In this study, goniothalamin, a natural occurring styryl-lactone extracted from *Goniothalamus macrophyllus,* was investigated for

cytotoxic properties against cervical cancer (HeLa), breast carcinoma (MCF-7) and colon cancer (HT29) cells as well as normal mouse fibroblast (3T3) using MTT assay. Fluorescence microscopy showed that GTN is able to induce apoptosis in HeLa cells in a time dependent manner.[34]

Sanjay patel et al.,(2009) The study was aimed to evaluation of the anticancer activity of the fruits of Solanum Nigrum on the HeLa cell line. The fruits of Solanum Nigrum methanolic extract were tested for its inhibitory effect on HeLa Cell Line. The percentage viability of the cell line was carried out by using Trypan blue dye exclusion method. The cytotoxicity of Solanum Nigrum on HeLa cell was evaluated by the SRB assay and MTT assay. Solanum Nigrum methanolic extract has significant cytotoxicity effect on HeLa Cell Line in concentration range between 10 mg/ml to 0.0196 mg/ml by using SRB assay and study also showed that inhibitory action on HeLa cell line in concentration range between 10 mg/ml to 0.0196 mg/ml by using MTT assay.[35]

Fluorouracil (5FU) :

Ali Alshehri et al.,(2012) reported the antiproliferative effect of *Cinchorium endivia L.* phenolic extracts on breast cancer cell line MCF-7. Anticancer activity of methanolic extract of root of *C.endivia, L* was evaluated on breast cancer cell line MCF-7 and was compared with anticancer standard drug 5 FU (5 Fluorouracil). MTT assay was used to study the cytotoxicity of the root extract and standard drug. IC50 value was found out to be 401µg/ml for root extract and 0.67 µg/ml for standard 5 FU.[36]

Kameshwaran et al., (2012) reported the anticancer activity of methanolic extract of *Tecoma stans* flowers. The anticancer activity of methanolic extract of *T.stans* was evaluated by both *in vitro* and *in vivo* methods. Cytotoxicity assay was performed with Vero and Hep2 cell lines using MTT assay and it was compared with standard 5

Flourouracil, whose cytotoxicity was found to be 85.78% in Vero cell line and 81.53% in Hep 2 cell line.[37]

P53:

Role of p53 in human cancers have been studied extensively in the past decades and restoring wild type p53 has been a major target in cancer therapy.

Anjana munshi et al.,(2009) reported the apoptotic effect of chloroform, n-hexane and methanolic extracts of *Nigella sativa* on HeLa cells which contained certain compounds which up regulate the expression of p53.[38]

Bcl2:

Gul Ozcan Arican.,(2012) reported the effect of *Astragalus L* root extract on proliferation of He La cells and Bcl-2 gene expressions. Cytotoxic effect of ethyl acetate extract of root was investigated using MTT assay and the extract was reported to possess cytotoxic effect on HeLa cells. Effect of root extract on gene expression rates involved in apoptosis was studied using RT-PCR technique. Anti apoptotic gene Bcl-2 was found to be expressed at a dose of 1 mg/ml for a treatment up to 24 hours and this expression was disappeared when the treatment was extended up to 48 hours.[39]

Microscopic Studies:

Shruti Nair et al.,(2011) reported the anticancer activity of alcoholic and aqueous extracts of *Moringa oleifera* on Hela cells. Anti proliferative effect was assessed by MTT assay and apoptosis of cancer cells were confirmed by DNA fragmentation test and ethidium bromide-acridine orange staining. It was reported that staining the cells which was treated with different concentrations of *Moringa oleifera* aqueous extracts

showed viable cells green with intact nuclei and non viable cells with bright orange chromatin.[40]

Apoptotic DNA Fragmentations:

Apoptosis also known as 'programmed cell death' is the elimination of no longer wanted cells in an active and inherently controlled manner. Characteristic of apoptotic process are:

- Cell and nuclear shrinkage
- Chromatin condensation
- Formation of apoptotic bodies[41]

DNA fragmentation in cells undergoing apoptosis is caused by enzyme namely Caspase Activated DNase or CAD, which is inhibited by another protein namely Inhibitor of Caspase Activated DNase or ICAD. During apoptosis CAD is activated by apoptotic effector caspase, caspase 3. CAD is usually complexed with ICAD in proliferating cells. In cells where apoptosis is induced, caspase 3 cleaves the CAD:ICAD complex thereby allowing CAD to cleave the chromosomal DNA into nucleosomal fragments leading to apoptosis. Many chemotherapeutic drugs like mitomicin C induce apoptosis by causing damage to nuclear DNA. Cleavage of chromosomal DNA into oligonucleosomal fragments is a late event of apoptosis.[42]

Kanimozhi D et al., DNA fragmentation was obtained by agarose gel electrophoresis of ethanolic extract of *Cynodon dactylon* with Hep-2, HeLa and Mcf-7 cancer cell lines. The DNA migrated as discrete bands which was compared to DNA markers, gave a ladder of approximately 200 base pair (bp). Such DNA ladders are considered to be a hall mark of apoptosis, continues smears may also indicate DNA fragmentation due to apoptosis. The ladder from DNA fragmentation catalyzed by an

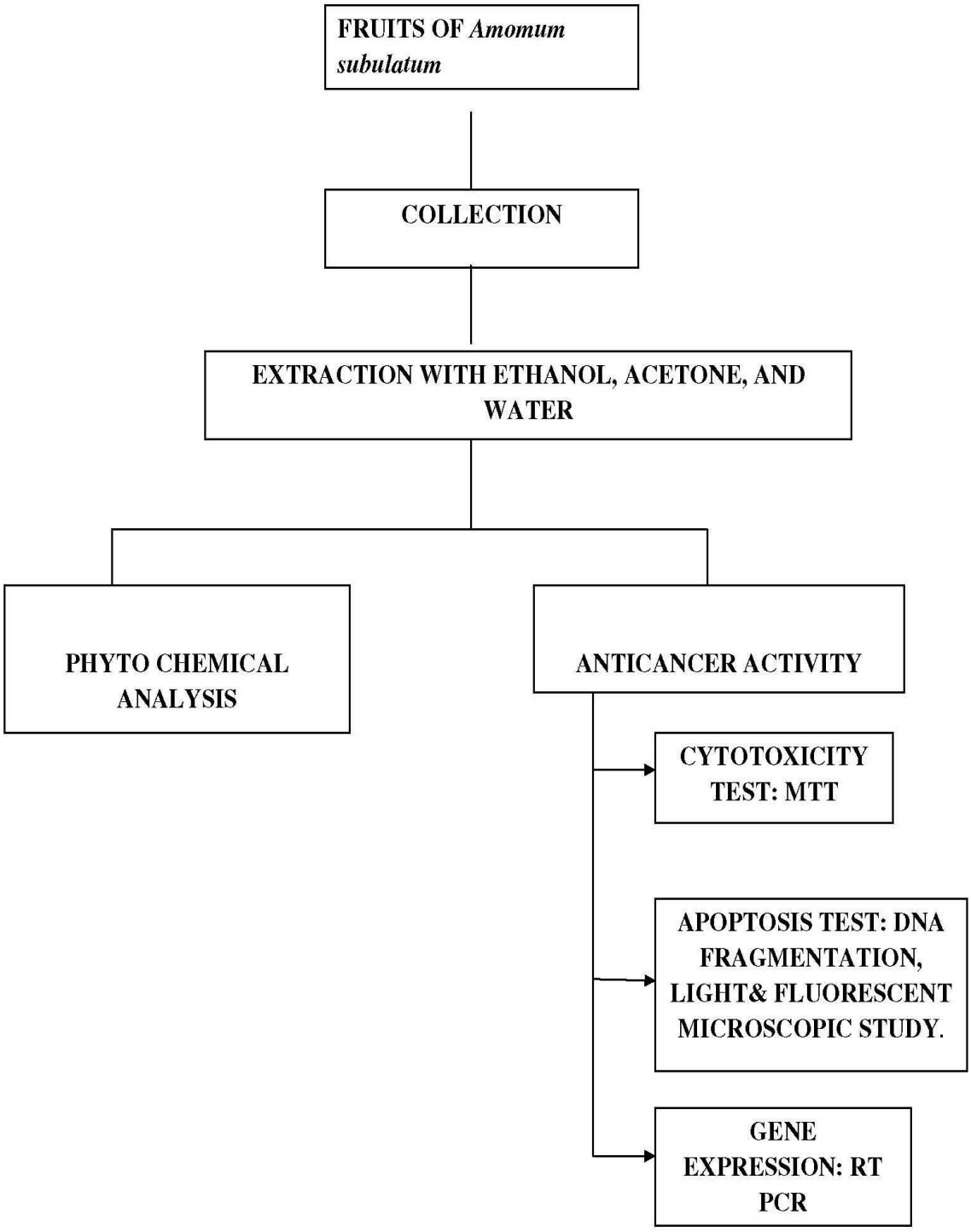
endogenous endonuclease that cleaves internucleosomal DNA to form ladder like bands of oligo nucleosome fragments. From this it is revealed that these DNA fragments (fig.3) shows that the ethanolic extract of Cynodon dactylon has anticancer activity in the Hep-2, HeLa and Mcf-7 cell lines.[43]

Real Time RT-PCR

Ali Alshehri (2012) reported the anti proliferative effect of *Cinchorium endivia L*, phenolic extract on cancer cell line HCT-116. The anticancer activity of the plant root extract was studied on colon cancer cell line HCT-116. Cytotoxicity was measured and compared with 5 FU. Gene expression levels of cancer markers p53, Bcl-2, TNF-α and immune response markers IL-2, 4 and 6 along with housekeeping gene primer GPDH were studied using real time RT- PCR , SYBR green model. It was reported that p53 expression was found to be same in control and treated cells. Bcl2 expression was low in treated cells when compared to control. TNF expression was found to be same in cells treated with 5 Fluorouracil and control, but higher in cells treated with plant extract.[44]

Ali A Alshatwi et al.,(2011) reported the anti tumour activity of methanolic extract of lemon fruit on MCF-7 breast cancer cell line. Apoptotic cell death was studied using TUNEL Assay. The expression levels of apoptotic genes was analyzed by reverse transcription PCR using a real time SYBR green gene expression assay kit. It was reported that p53 and caspase 3 expression was increased which lead to apoptosis. Expression levels of pro apoptotic gene bax was increased and anti apoptotic gene Bcl2 was decreased after treatment with lemon extract.[45]

PLAN OF WORK:


FRUITS OF *Amomum subulatum*
COLLECTION
EXTRACTION WITH ETHANOL, ACETONE, AND WATER
PHYTO CHEMICAL ANALYSIS
ANTICANCER ACTIVITY
CYTOTOXICITY TEST: MTT
APOPTOSIS TEST: DNA FRAGMENTATION, LIGHT& FLUORESCENT MICROSCOPIC STUDY.
GENE EXPRESSION: RT PCR

4.0 METHODOLOGY

4.1 EXTRACTION

It is defined as separation of medicinally active parts of plant using selective solvents through standard procedure. Extraction procedure was carried out in Institute of Pharmacology, Madras Medical College, Chennai.

4.2 PLANT PARTS USED:

The fruits of *Amomum subulatum* was used for extraction which was collected in month of July 2013 at Tirunelveli. It was examined and authenticated by Mr. V. Chelladurai, officer- Botany (scientist-c) (retd.), Central Council for Research in Ayurveda & Siddha, Govt. of India.

4.3 SOLVENTS USED:

Based on polarity various solvents were used such as ethanol, acetone, and water.

4.4 TYPE OF EXTRACTION

Hot continuous (soxhlet) extraction method.

APPARATUS DESCRIPTION

The apparatus consists of body of the extractor (thimble) attached with side tube, siphon tube, lower end attached with distillation flask, mouth of the extractor is fixed to the condenser by standard joints.

PRINCIPLE

It is a process of continuous extraction method in which the solvent can be circulated through the extractor for several times. The vapours of solvent are taken to the condenser and the condensed liquid is returned to the extract for continuous extraction.

PROCEDURE

50 grams of fruit was packed into soxhlet apparatus and was subjected to extraction sequentially with 500ml of ethanol, acetone, and water. The extraction was continued until the colour of the solvent in the siphon tube became colourless.

Extracts of acetone and ethanol were subjected to evaporation at room temperature till a semisolid mass was obtained. Aqueous extract was subjected to lyophilisation with the help of lyophiliser to a semisolid mass.

4.5 PHYTOCHEMICAL ANALYSIS:[46]

The freshly prepared extracts of acetone, ethanol and water were subjected to phytochemical screening for the presence or absence of active phytochemical constituents by following methods.

Test for Steroids:

Salkowskis test: Crude extract was mixed with 2ml of chloroform. Then 2ml of conc. Sulphuric acid was added carefully and shaken gently. Appearance of reddish brown colour ring indicated the presence of steroids

Test for Flavanoids:

Shinoda test: Crude extract was treated with 5 ml 95% ethanol, few drops concentrated hydrochloric acid and 5 grams magnesium turnings, appearance of pink colour indicated the presence of steroid

Lead acetate test: Crude extract was treated with few drops of lead acetate solution. Appearance of yellow colour precipitate indicate the presence of flavanoids

Alkaline reagent test: Crude extract was treated with few drops of sodium hydroxide solution. Formation of intense yellow color, which becomes colorless on addition of dilute acid, indicates the presence of flavanoids.

Test for terpenoids:

5 ml of each extract was mixed in 2 ml of chloroform. 3 ml of concentrated H2SO4 was then added to form a layer. A reddishbrown precipitate colouration at the interface formed indicated the presence of terpenoids.

Test for Proteins:

Millions test: Crude extract was mixed with 2ml of millions reagent. Appearance of white precipitate which turns red on gentle heating, indicates the presence of proteins.

Test for Glycosides:

Liebermann's test: Crude extract was mixed with 2ml of chloroform and 2ml of acetic acid. Mixture was cooled in ice and conc. sulphuric acid was added. Colour change from violet to blue to green indicates the presence of steroidal nucleus

Test for Carbohydrates:

Fehling's test: Crude extract was treated with equal volume of Fehling A and Fehling B reagents and mixed together and gently boiled. Appearance of brick red precipitate at the bottom of the test tube indicate the presence of reducing sugars

Test for phenols and tannins:

Crude extract was mixed with 2ml of 2% solution of ferric chloride. Appearance of violet colour indicates the presence of phenolic compounds and tannins. Crude extract was dissolved in water and treated with 10% of lead acetate solution, appearance of white precipitate indicate the presence of tannins and phenolic compounds.

Test for alkaloids:

Crude extract was treated with few drops of dilute hydrochloric acid and filtered. The filterate was tested with various alkaloidal reagents such as

Mayer's reagent – Cream precipitate

Dragendroff's reagent –Orange brown precipitate

Wagner's reagent–Reddish brown precipitate

4.6 PREPARATION OF MEDIA

Definition

Media is a nutritional supplementation essential for maintenance, growth and division of cells in-vitro.

Materials required

1. Minimum essential medium (MEM) in powder form.
2. Penicillin (100 IU/ml)
3. Streptomycin (100 μg/ml)
4. Phenol red
5. Amphotericin B
6. L-glutamine (3%)
7. Foetal bovine serum (FBS) 10%
8. Sodium bicarbonate (7.5 %)

Procedure:

- Powder form of MEM was dissolved in one liter of pre sterilized Millipore distilled water, mixed well and closed.
- The medium was sterilized for 15 minutes at 121°c.

- ❖ 856 ml of sterilized MEM was taken in a 1000 ml flask and to it each ml of penicillin, streptomycin, phenol red and amphotericin B was added and mixed well.
- ❖ 10 ml of 3% L-glutamine, 100 ml of FBS and 30 ml of 7.5% sodium bicarbonate was added to make up the volume to 1000 ml.
- ❖ pH was adjusted to 7.2 – 7.4 and the medium was stored for two days at 37°c and the pH was checked and transferred to the refrigerator.

4.7 CELL LINES AND CULTURE

HeLa Cell line was obtained from Tamil Nadu Veterinary College, Chennai. The cells were maintained in Minimal Essential Media supplemented with 10% FBS, penicillin (100 U/ml), and streptomycin (100 µg/ml) in a humidified atmosphere of 50 µg/ml CO_2 at 37 °C.

4.8 SUB-CULTURING ADHERENT CELLS:[47]

Materials Required:

1. Incubator (37°c, 5% CO2)
2. Laminar flow hood
3. Minimum essential medium
4. TPVG solution (Trypsin 2%, PBS – pH 7.4, Versene/EDTA- 0.2%, glucose10%)
5. Tissue culture treated culture flask
6. Micropipette
7. Falcon tubes- conical
8. Inverted microscope

Procedure:

- The culture flask containing the adherent cells (primary culture) was removed from the incubator.
- The culture flask was transferred aseptically to the bio safety cabinet along with minimum essential medium and TPVG solution after confirming its 90% confluency with the help of inverted microscope.
- The primary culture was passaged /sub cultured to required number of culture flasks for

  further study.
- The cap of the culture flask was opened and with the help of micropipette the medium was aspirated from the culture flask and discarded.
- 200µl of TPVG solution was added to the culture flask and rinsed well to remove any adhering medium and later the solution was aspirated and discarded.
- 500-1000µl of TPVG solution was again added to the culture flask and the solution was spread evenly to cover the entire surface of the culture flask.
- The flask was then incubated for 5 minutes for detachment of adhered cells from the monolayer (cell detachment can be viewed and confirmed under microscope after 5 minutes of incubation).
- After detachment of cells from the monolayer, the culture flask was transferred back to the bio safety cabinet. The detached cells were suspended completely in the TPVG solution by re suspending it with the help of micropipette.
- The TPVG solution containing the detached cells were aspirated from the culture flask and distributed into 7 new tissue culture treated culture flasks for further studies.

- ❖ 5ml of fresh minimum essential medium was added to all 7 culture flask containing the TPVG solution with suspended cells, such that it covers the entire surface of the flask.

4.9 CYTOTOXICITY STUDY

MTT ASSAY:[48]

It is a universally accepted in-vitro method for screening the drugs having cytotoxic activity. It was described by Mosmamm (1983) & Monks (1991). This assay is used to determine the IC50 of drugs or extracts.

PRINCIPLE

The Tetrazolium salt, 3,-(4,5- dimethyl thiazol-2-yl)-2,5,diphenyl tetrazolium bromide is reduced into blue formazan product by the mitochondrial dehydrogenase enzyme of live or metabolically active cells.

The intensity of blue or purple colored formazan produced is directly proportional to cell viability.

Materials required

1. **Plant extracts**

   Ethanol (1000, 500, 250, 125, 62.5, 31.2, 15.6, 7.8)

2. **Reagents**

   DPBS

   TPVG

   MEM

   Tryphan blue

   MTT dye(5mg/ml)

   DMSO(0.1% v/v)

3. **Other materials**

MTT plate with plate with lid

Sterile pipette, tips, glove, mask

Aluminium foil

Incubator

Inverted microscope

Falcon tubes

Haemocytometer, cover slip.

PROCEDURE

- Culture flask containing the adherent cells were taken from the incubator and checked for its confluency.
- After confirming the confluency, the adherent monolayer cells were detached to form a cell suspension by adding an ml of TPVG solution and incubating it for 5 minutes.
- After incubation, the TPVG solution containing cells were aspirated from the culture flask and transferred to the falcon tubes and was subjected to centrifugation at 10000 rpm for 5 minutes.
- After centrifugation, supernatant was discarded and 1 ml of medium was added to the pellet of cells which have sedimented at the bottom of the tube and was re suspended well in the medium with the help of micropipette.
- 10µl of this cell suspension was mixed well with 10µl of tryphan blue.

Cell counting:

- After placing the cell suspension along with tryphan blue in haemocytometer, the numbers of cells in four corner quadrants were counted.

- Numbers of cells in left and right top quadrant were found to be 126 and 147respectively. Numbers of cells in left and right bottom quadrant were found to be 138 and 142 respectively.
- Total number of cells/ml were calculated as follows:

$$\frac{126+147+138+142\times2\times10^{4}}{4} = 276.5\times10^{4\text{cells/ml}}$$

- 276.5×10^{4} cells were present in 1 ml of cell suspension; we need to plate 26 wells each with 100 µl containing 10000 cells/well for maintaining triplicate condition for control, standard and test extracts.
- Therefore, 276.5 x 10^{4} cells were present in 1000 µl. 10000 cells were present in x µl.

$$X = \frac{10000 \times 1000}{276.5\times10^{4}} = 3.6\mu l$$

- 3.6 µl of cell suspension should be mixed with 96.4 µl of medium for plating one well. In order to plate 26 wells, 93.6 µl of cell suspension was mixed with 2506 µl of medium to get a concentration of 10000 cells / 100 µl for plating the wells.
- 100 µl of this cell suspension were seeded into 26 wells of 96 well plate, and the plate was incubated for 24 hours at 37°c, 5% CO2 for attachment of the cells.
- After 24 hours the seeded cells in the 96 well plate were treated with serial concentration of plant extract and the standard drug 5FU.
- Plant extracts of acetone, ethanol and aqueous and standard drug were initially dissolved in 0.1% v/v DMSO and further diluted in serum free medium to get a desired concentration.
- 100 µl of each plant extract and standard concentration were added to the 96 well plates to get a final concentration of 1000 µg/ml, 500 µg/ml, 250 µg/ml, 62.5

μg/ml, 31.2 μg/ml and 15.6 μg/ml for plant extracts and 1 μg/ml, 2.5 μg/ml, 5 μg/ml and 10 μg/ml for standard drug.

- ❖ Final volume in each well was 200 μl and the plates were incubated again for 48 hours.
- ❖ Triplicate was maintained for all concentrations and the medium without samples were taken as control.
- ❖ By the help of Inverted microscope Toxicity is observed.

4.10 Extraction of DNA, RNA using Genei TRI Solution:

Extraction of DNA, RNA from the cells which was treated with IC_{50} concentration of Ethanolic extract, IC_{50} concentration of 5 Fluorouracil and from cells without any treatment were extracted as per Genei TRI Soln Protocol.[49] Fully grown cells or 90% confluency reached 3 subcultured or passaged flask is taken. The medium inside the flask is aspirated and decanted while cells adhered to the monolayer was kept intact and the flasks were subjected to drug treatment.

- ❖ Flask 1 is replaced with the fresh medium without any test or standard drug and it served as Control.
- ❖ Flask 2 is treated with IC50 concentration of the effective ethanolic extract.
- ❖ Flask 3 is treated with IC50 concentration of standard 5 fluorouracil (IC50 confirmed from MTT Assay).

All the three flasks were incubated for 48 hours at 37°c and 5% CO2. After the incubation period, the medium inside the flasks were removed and the adhered cells were disturbed by treatment with TPVG solution and the TPVG solution containing the suspended cells of three flasks were stored in refrigerator for further processing

Extraction of RNA :

Materials Required:

1. Chloroform
2. Isopropyl Alcohol
3. 75% Ethanol
4. RNase free water

Steps Involved:

1. Homogenisation
2. Phase Separation
3. RNA Precipitation
4. RNA Wash
5. Resuspending RNA

Procedure:

Homogenisation:

- Cells were pelleted by centrifugation at 1200 rpm for 5 minutes.
- The supernatant was decanted and 1 ml of TRI solution was added and mixed by repetitive pipetting

Phase Separation:

- The samples were incubated for 5 minutes at room temperature (without exceeding 30°c) to completely dissociate nucleoprotein complex.
- 0.2 ml of chloroform per 1 ml of TRI Solution was added, mixed thoroughly and subjected to incubation at room temperature (without exceeding 30°c) for 2-3 minutes.

- The samples were centrifuged at not more than 12000 rpm for 15 minutes at 2-8°c and the mixture thus obtained contained a lower phenol chloroform phase, interphase and colourless upper aqueous phase.
- RNA was present in upper aqueous phase.
- Volume of aqueous phase obtained was about 60% of volume of TRI Solution reagent used for homogenization.

RNA Precipitation:

- Aqueous phase was transferred to fresh 1.5 ml vial.
- 0.5 ml of isopropanol alcohol per 1 ml of TRI Solution was used for initial homogenization.
- Samples were incubated for 10 minutes at room temperature and were subjected to centrifugation at 12000 rpm for 10 minutes at 2-8°c.

RNA Wash:

- Supernatant was decanted and the RNA precipitated as gel like pellet on the sides of 1.5 ml vial.
- RNA pellet was washed once with 75% ethanol, at least 1ml of 75% ethanol was used per ml of TRI Solution reagent used for initial homogenization.
- Solution was centrifuged at 10000 rpm for 10 minutes at 2-8°c

Resuspending RNA:

- RNA pellet was air dried and re-suspended in100µl RNase free water by passing solution few times through pipette tip gently

Extraction of DNA:

Materials required:

1. Ethanol (100%)
2. 75% Ethanol
3. 0.1 M sodium citrate in 10% ethanol.
4. 8mM Sodium hydroxide.
5. Wash Buffer : 0.1 N Sodium citrate in 10% ethanol.

Steps involved:

1. DNA Precipitation
2. DNA Wash
3. Re suspending the DNA pellet.

Procedure:

DNA Precipitation:

- 0.3 ml of 100% ethanol was added per 1 ml of TRI Solution used for homogenization to the inter phase and organic phase which was obtained during phase separation in RNA extraction.
- Samples were stored at room temperature without exceeding 30°c for 2-3 minutes.
- Samples were centrifuged at 5000 rpm for 10 minutes at 2-8°c and DNA was obtained as a pellet.

DNA Wash:

- DNA pellet obtained was washed with wash buffer per 1 ml TRI Solution used.
- At each wash pellet was stored in wash solution for 30 minutes at room temperature without exceeding 30°c with periodic mixing.

- Samples were centrifuged at 5000 rpm for 10 minutes at 2-8°c and the wash step was repeated twice.
- Following wash, DNA pellet was suspended in 2 ml of 75% ethanol and kept for twenty minutes at room temperature and mixed periodically.
- Samples were centrifuged at 5000 rpm for 10 minutes at 2-8°c.

Re-suspending the DNA Pellet:

- DNA pellet was air dried for 5-10 minutes.
- DNA pellet was re-suspended in 300µl to 600µl of 8mM sodium hydroxide so that the concentration of DNA was 0.2 - 0.3µg/ml and samples were incubated at room temperature for 15-20 minutes

4.11 MICROSCOPIC STUDIES:[50]

Light microscopic study

Fluorescent microscopic study.

Light microscopic studies:

- Morphological changes in the cancer cells before and after drug treatment can be studied with the help of Light microscope.
- Fully grown or 90% confluency reached subcultured or passaged flask was taken.
- The morphology of the cancer cells in the flask before drug treatment were observed under inverted microscope and photographed.
- The medium inside the flask was aspirated and decanted without disturbing the adhered monolayer of cells.
- The culture flask was then treated with IC50 concentration of effective plant extract such that it covers the entire surface area

- The flask was then incubated for 48 hours at 5% CO2 and 37°c.
- After incubation period, the flask was taken and observed under inverted microscope and the morphological changes were studied and photographed.
- The morphological changes of the cancer cells before and after drug treatment were evaluated.

Flourescent microscopic studies:

- Fluorescent microscopy was used to study the viability of cells as well as nuclei and chromatin condensation with the help of fluorescent binding dye.
- Fully grown or 90% confluency reached two subcultured or passaged flask was taken.
- Flask 1 was used for studying the control cancer cells and flask 2 was used for studying the extract treated cancer cells.
- From flask 1 the adhered cells were detached with the help of TPVG solution and the TPVG solution containing the cells were centrifuged and the cells were pelleted.
- The pellet of cells were then resuspended in phosphate buffer saline of pH 7.4. 100 µl of this cell suspension was introduced into microscopic slide along with equal mixture of acridine orange and ethidium bromide for staining.
- The cells were then viewed under fluorescent microscope and the viability and nuclear changes were studied and photographed.
- In flask 2, the medium was aspirated and decanted without disturbing the adhered monolayer of cells.
- Flask was then treated with IC50 concentration of effective plant extract such that it covers the entire surface area.
- The flask was then incubated for 48 hours at 5% CO2 and 37°c.

- ❖ After incubation period, the flask was taken and the cells were pelleted and the same procedure was followed as in flask 1.

4.12 APOPTOTIC DNA FRAGMENTATION:[51]

Apoptosis is characterized by cleavage of chromosomal DNA into oligonucleosomal fragments. Irregularities in apoptosis have paved way for many diseases like cancer, autoimmune disease and neuronal degeneration. This cleavage of DNA or its fragmentation can be visualized by DNA laddering assay. Cleavage of chromosomal DNA into oligonucleosomal fragments is a hallmark of apoptosis. This fragmentation of DNA in cancer cells after treatment with standard drug or test extract can be studied with the help of Agarose Gel Electrophoresis. Electrophoresis is a method of separating substances based on the rate of movement under the influence of electric field.

Materials required:

1. Agarose
2. 5X TAE buffer
3. 6X loading dye
4. DNA ladder
5. Electrophoresis chamber
6. Gel casting tray and comb
7. Ethidium bromide
8. Microwave oven
9. Conical flask
10. Sterile tips and pipettes

Procedure:

The steps involved are:

- Preparation of Agarose gel slab
- Loading of samples
- Running the gel

Preparation of Agarose gel slab:

- Gel casting tray and comb was wiped with ethanol and dried. The two open ends of the tray were sealed with tape.
- Place the combs in the gel casting tray.
- 1.2 grams of agarose powder was weighed and transferred to 500 ml conical flask containing 100 ml of TAE buffer.
- Agarose was melted using microwave oven until the solution became clear.
- The solution was cooled to about 50 - 55°c by swirling the flask occasionally or placing it in water bath.
- 3µl of ethidium bromide was added to the solution and mixed well.
- The gel casting tray along with comb was kept horizontally on a flat even surface.
- Agarose solution was poured evenly on the casting tray such that it covers the entire surface evenly without any bubble formation and was allowed to cool and solidify into a gel slab.
- After solidification, remove the tapes from both ends of the tray and place the gel in electrophoresis chamber and add enough TAE buffer so that there is 2-3 mm of buffer over the gel and carefully pull out the comb out of the tray to form wells.

Loading of samples:

- DNA which was extracted from the control, standard and extract treated cancer cells.
- Based on the quantification, equal quantity of DNA was taken and mixed with required TAE buffer to make a final volume of 10µl each for a sample.
- 2 µl of 6X loading dye was added to the DNA sample and mixed well.
- 10µl of 100 base pair standard DNA ladder and 12µl of DNA samples were loaded carefully in the submerged wells of gel slab in the electrophoresis chamber

Running the Gel:

- Lid was placed on the gel box and the electrodes were connected.
- Electrode wires were connected to power supply and power supply of about 80 volts were applied.
- Power supply to the electrophoresis chamber was ensured until the blue dye approached the end of the gel.
- Later power supply was cut off, wires were disconnected and the lid was removed.
- Gel tray was carefully taken out and the gel was placed inside the Gel photostation and photographed in presence of UV light

4.13 REAL TIME REVERSE TRANSCRIPTASE POLYMERASE CHAIN REACTION:[52]

Real time reverse transcriptase polymerase chain reaction is abbreviated as qRT-PCR. It is a technique where expression of RNA is studied by converting it into cDNA with the help of enzyme reverse transcriptase and quantitatively measuring the amount of

amplified target sequence from entire cDNA using fluorescent dye SYBR green in real time. Upon binding with DNA, SYBR green dye used will emit fluorescence and the fluorescence intensity is directly proportional to number of DNA copies or expression produced. The fluorescence which is emitted is analysed by detector with the help of LED source and it gives the relative expression of genes. The procedure was carried out as per Step 1 plus ABI protocol.

Procedure:

A) Complementary DNA Synthesis:

Materials Required:

1. 5X buffer
2. 10mm dNTPs
3. Hexamer primer
4. Extracted RNA
5. Thermal cycler
6. Reverse transcriptase
7. DTT

Procedure:

- In 200µl eppendorf tubes, 5µl of 5X buffer, 2µl of 10mm DNTPs and 1.5µl of hexamer primer was added.
- Later 15µl of extracted RNA was added and the eppendorf tubes were kept in thermal cycler at 70°c for 5 minutes to separate the false double stranded RNA.
- The tubes were taken out and immediately cooled with ice to prevent binding of false double stranded RNA again.

- 1.5µl of Reverse transcriptase and 1µl of DTT was added to the tube and spun for few seconds.
- It was then placed in thermal cycler , 25°c for 5 minutes, for binding of hexamer, followed by 42°c for 45 minutes for cDNA synthesis, followed by 85°c for 5 minutes for denaturation of remaining unconverted RNA's and finally at 4°c for 5 minutes

B) Primer synthesis:

The primers synthesized were P53, Bcl2, along with house keeping gene GPDH. The primers were synthesized by Geno Rime with the help of Primer express software with the available primer sequence.

The concentration of primers synthesized were 100pM/µl. It was diluted in the ratio of 1: 10 with water to get a concentration of 10pM/µl

C) Real time PCR:

The materials required are

- ✓ 25µl of SYBR green
- ✓ RT mix
- ✓ 5µl of cDNA
- ✓ 2µl of 25 pM/µl forward primer
- ✓ 2µl of 25 pM/µl reverse primer
- ✓ 16µl PCR grade water.

- For a total of 50 µl reaction, the above mentioned mixtures were added into eppendorf tubes and they were placed in real time PCR instrument and the program was set as follows:

- ✓ Step 1: Pre denaturation at 95˚C for 1 minute
- ✓ Step 2: Denaturation at 95˚C for 15 seconds
- ✓ Step 3: Annealing at 60˚C for 15 seconds
- ✓ Step 4: Extension at 72˚C for 45 seconds
- ✓ Step 2 to step 4 repeated for 40 cycles.

❖ The relative expression of genes was analyzed and interpreted and by Applied Biosystem Software.

5. RESULTS

Table 1. Phytochemical analysis of various extracts of fruits of *Amomum subulatum*.

Constituents	Ethanol extract	Acetone extract	Aqueous extract
Proteins	Present	Present	Present
Carbohydrates	Present	Present	Present
Glycosides	Present	Present	Present
Triterpenoids	Present	Absent	Absent
Flavanoids	Present	Absent	Absent
Alkaloids	Absent	Absent	Absent
Steroids	Present	Present	Present
Phenols and tannins	Present	Present	Present

Table 2. Percentage yield of extracts

Extract	% yield
Ethanol	5.20
Acetone	3.45
Aqueous	6.40

5.1 CYTOTOXICITY TEST

MTT assay was carried out with ethanolic extract of *Amomum subulatum* and with 5 Fluorouracil and the results are shown in Table 3 & 4.

Table 3. MTT assay of ethanolic extract of *Amomum subulatum*

S.No	Concentration (µg/ml)	Dilutions	Absorbance (O.D)	Cell viability (%)
1	1000	-	0.07	11.86
2	500	1:1	0.13	22.03
3	250	1:2	0.19	32.20
4	125	1:4	0.22	37.28
5	62.5	1:8	0.28	47.45
6	31.2	1:16	0.34	57.62
7	15.6	1:32	0.39	66.10
8	7.8	1:64	0.44	74.57
9	**Cell control**	**-**	**0.59**	**100**

IC50 concentration and Percentage cell viability

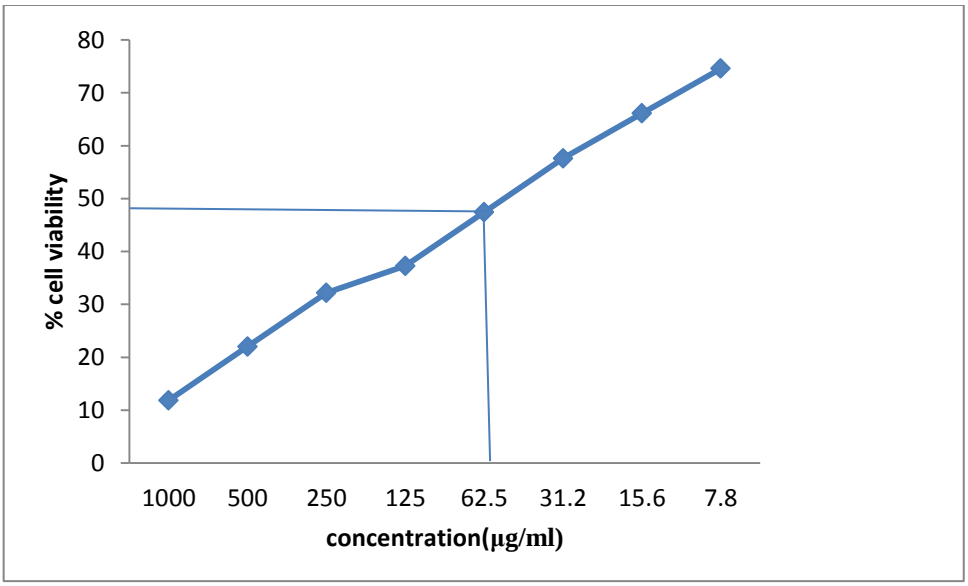


Figure 3. Graphical representation of % cell viability vs concentration (µg/ml) of ethanolic extract of *Amomum subulatum.*

IC50 concentration and % cell viability of 5 Fluorouracil

Table 4. MTT Assay of 5 Fluorouracil

S.No	Concentration (µg/ml)	Dilutions	Absorbance (O.D)	Cell viability (%)
1	1000	-	0.01	1.85185185
2	500	1:01	0.03	5.55555556
3	250	1:02	0.06	11.1111111
4	125	1:04	0.1	18.5185185
5	62.5	1:08	0.13	24.0740741
6	31.2	1:16	0.17	31.4814815
7	15.6	1:32	0.2	37.037037
8	7.8	1 64	0.28	51.8518519
9	Cell control	-	0.54	100

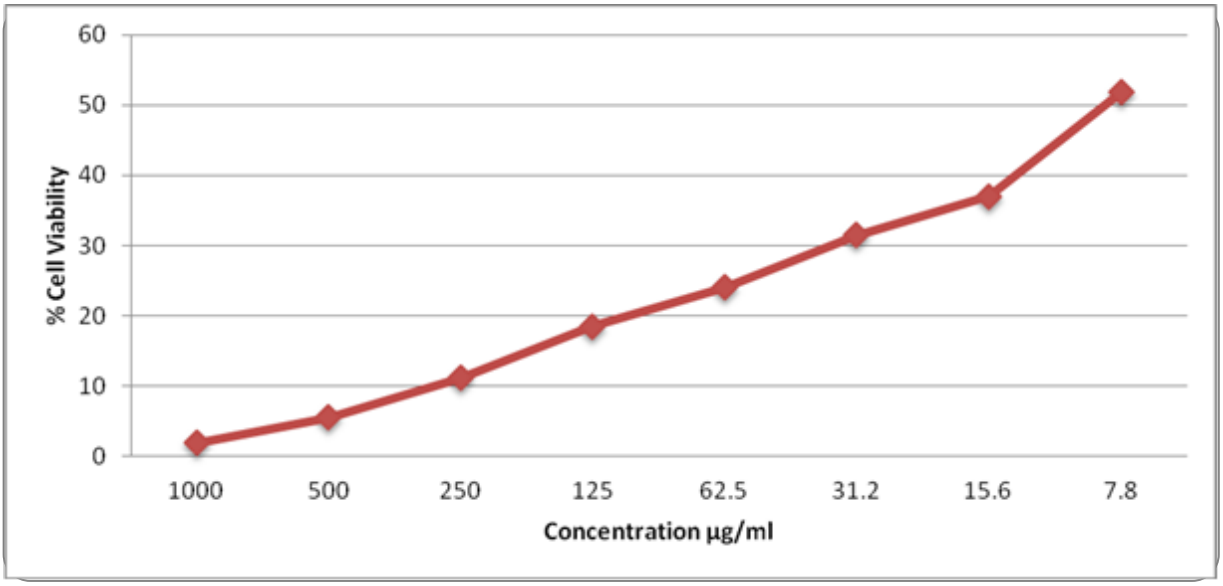
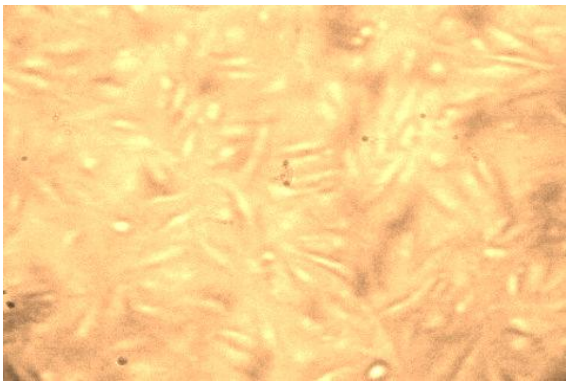


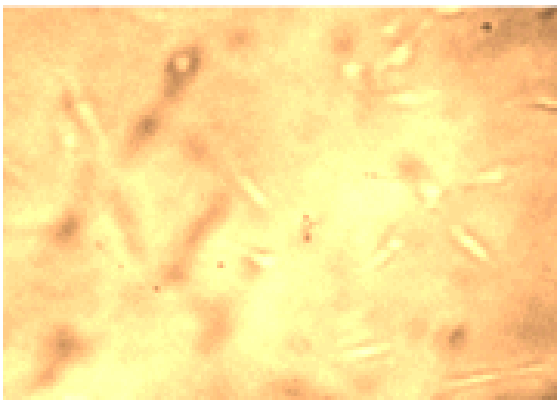
Figure 4: Graphical representation of % cell viability vs Concentration in µg/ml of 5 Fluorouracil

Anticancer effect of Sample *on HeLa* Cell line using microscope

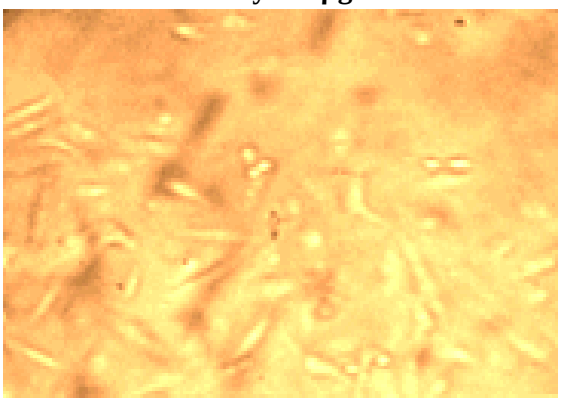
Normal *HeLa* cell line



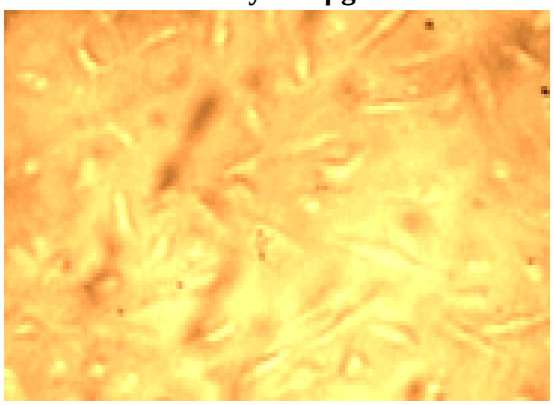
Toxicity-1000µg/ml



Toxicity-125µg/ml



Toxicity-62.5µg/ml



Toxicity-31.2µg/ml

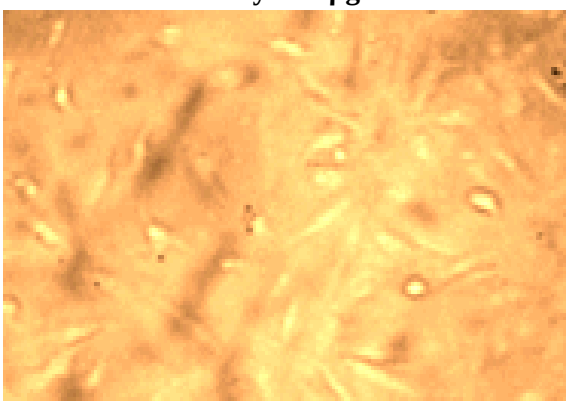


Figure 5. When HeLa cell line was treated with plant extract the cell death was more at 1000µg/ml and the least was at the concentration of 31.2µg/ml

5.2 MICROSCOPIC STUDIES FOR APOPTOSIS:

Light microscopic observation:

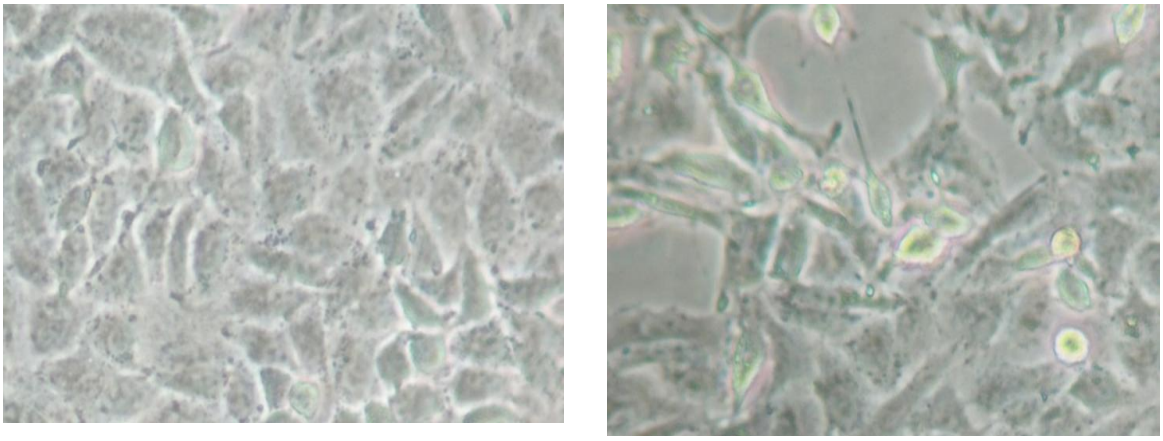
- Destruction of monolayer was observed in cells treated with IC50 concentration of ethanolic extract of *amomum subulatum*. The observations were compared with the cells without any treatment.
- Reduction of HeLa cell population and detachment of dead cells were observed in treated cells when compared to control cells.
- Loss of integrity of the membrane resulting in crooked and vesicle shape of the membrane, reduction in cell volume, cell shrinkage and chromatin condensation were observed in cells treated with extract when compared to control.

Flourescence microscopic observation:

- Control cells without any drug or extract treated were bright green in colour.
- Cells treated with ethyl acetate extract of *Amomum subulatum* were bright orange in colour with loss of membrane integrity and cytoplasmic contents leaking out of the cell.

The observations of light microscopic studies and fluorescent microscopic studies are depicted in figures 6 and 7

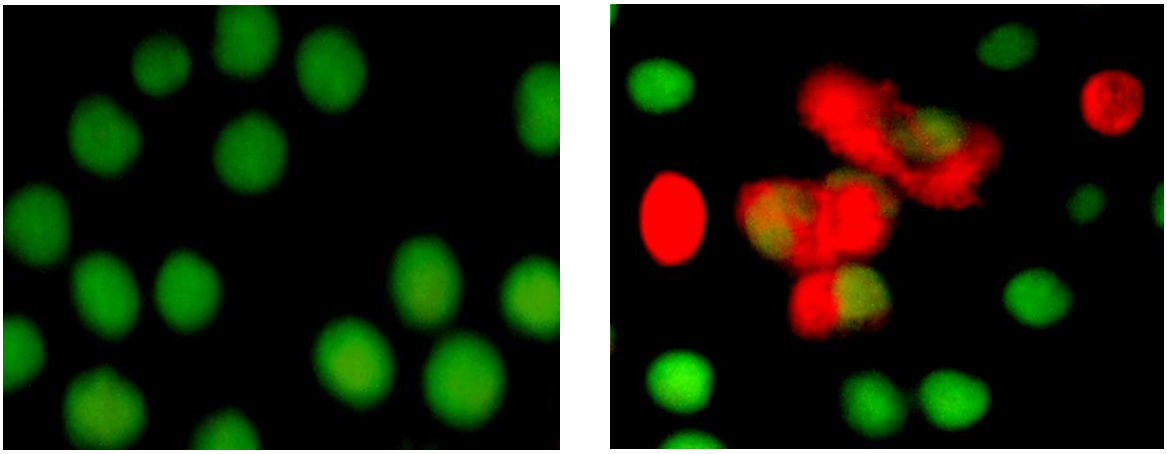
Figure 6 . Light Microscopic study images



A) HeLa cells – Control

B) HeLa cells after treatment with ethanolic extract of *Amomum subulatum* indicating decrease in cell population, chromatin condensation and destruction of monolayer

Figure 7. Flourescent Microscopic Images



A). HeLa cells – Control, indicting viable cells stained green in colour

B). HeLa cells after treatment with Ethanolic extract of *Amomum subulatum* showing dead cells stained orange in colour with loss of membrane integrity and cytoplasmic contents leaking out of the cell.

5.3 DNA Fragmentation:

- In the control HeLa cells, there was no fragmentation observed in agarose gel. Fragmentation was observed in HeLa cells treated with IC50 concentration of standard 5 fluorouracil and ethanolic extract of *Amomum subulatum.*
- This fragmentation of DNA in ethanolic extract treated cells indicated the characteristics of apoptotic cells.
- Results obtained in fragmentation studies are shown in figure 8.

Agarose gel electrophoresis

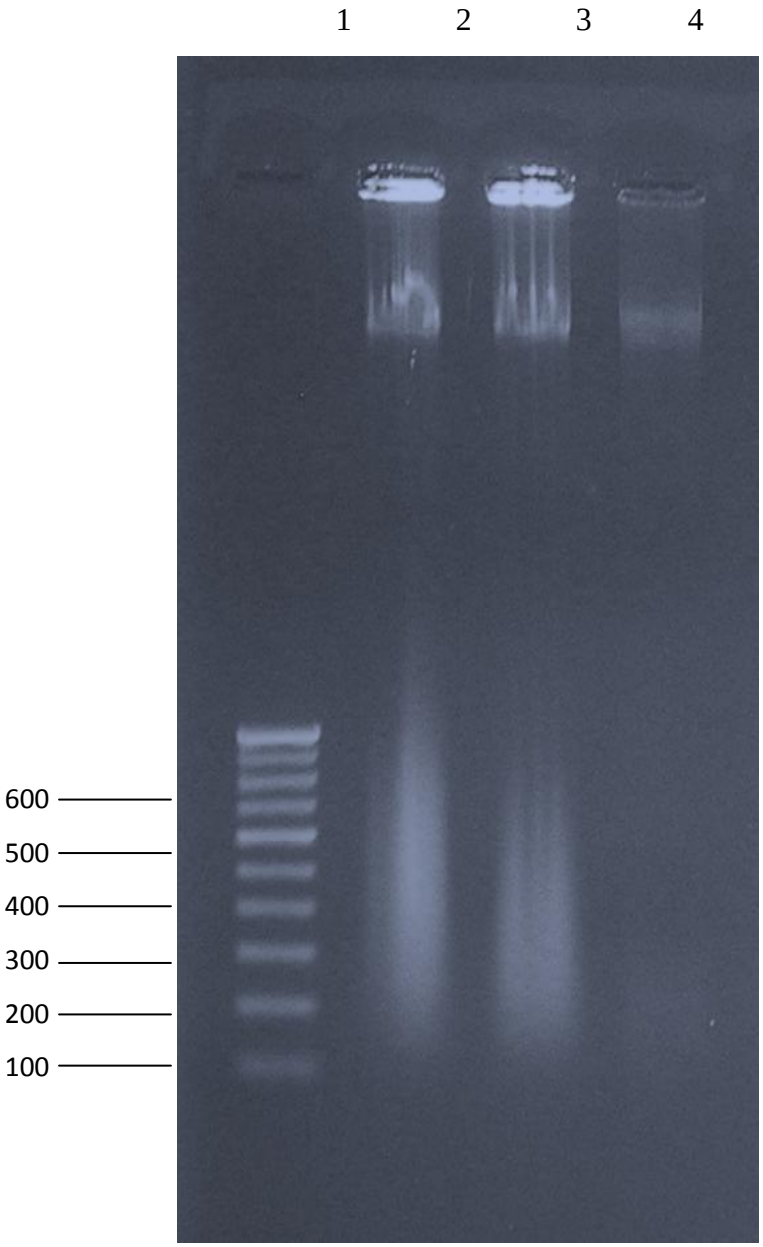


Figure 8. DNA Fragmentation in HeLa cells.

DNA Fragmentation in HeLa cells.

Lane 1: 100 base pair DNA marker

Lane 2: HeLa cells treated with 5 Fluorouracil

Lane 3: HeLa cells treated with ethanolic extract of *Amomun subulatum*

Lane 4: HeLa cells without any treatment.

5.4 Real time PCR :

The expression levels of p53, Bcl-2 were studied using RT-PCR and the results are shown in **Figure 9 and 11.**

P_{53} Gene in *HeLa* Cell Line by gel electrophoresis

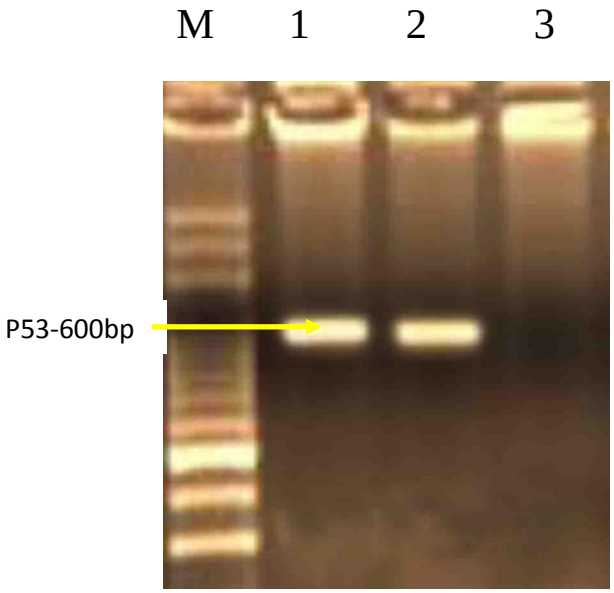


FIGURE 9. P53 gene expression

Figure 9: M- 100 bp DNA Ladder, Lane 1 and 2 : Samples in duplicate

Table 5. Expression levels of p53

P 53	Relative Quantification (ng/µl)	Standard error
Control	2.25	0.1
5 fluorouracil	6.15	0.12
Ethanolic extract	3.62	0.09

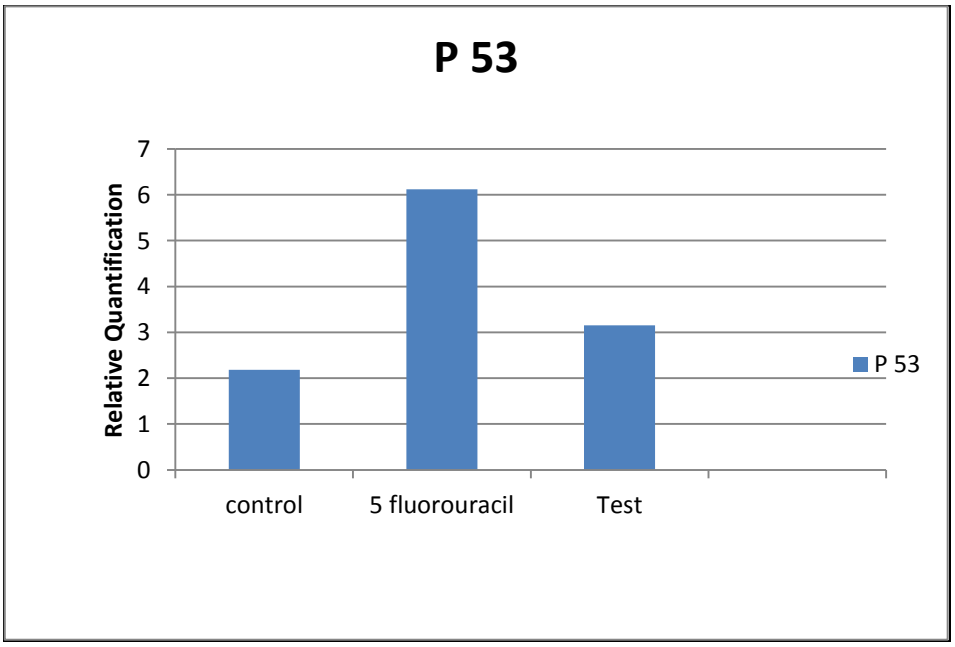


Figure 10. Graphical representation of p53 gene expression

BCl_2 Gene in *HeLa* Cell Line by gel electrophoresis

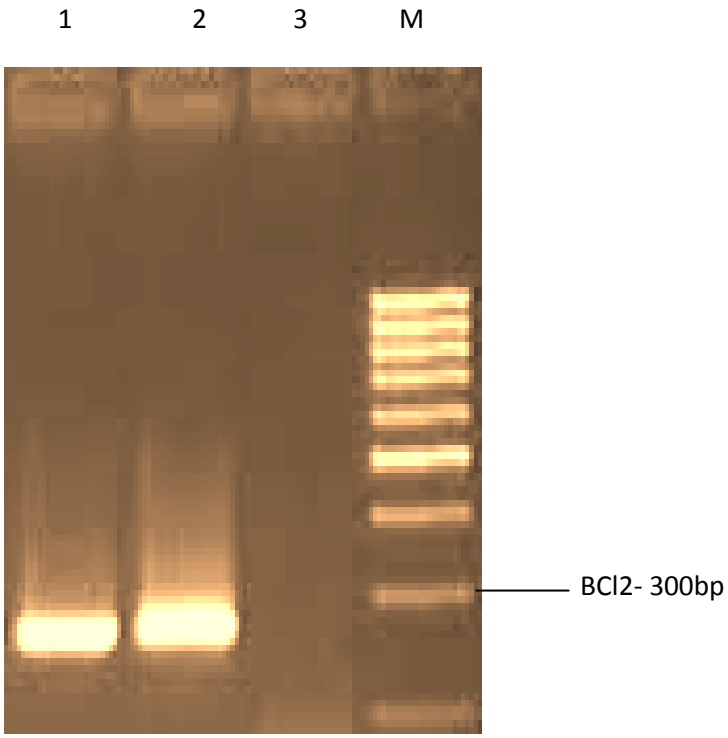


Figure 11: M- 100 bp DNA Ladder, Lane 1 and 2 : Samples in duplicate

Table 6- Expression levels of Bcl-2

Bcl2	Relative Quantification (ng/µl)	Standard error
Control	6.68	0.106
5 Fluorouracil	2.15	0.096
Ethanolic extract	3.84	0.131

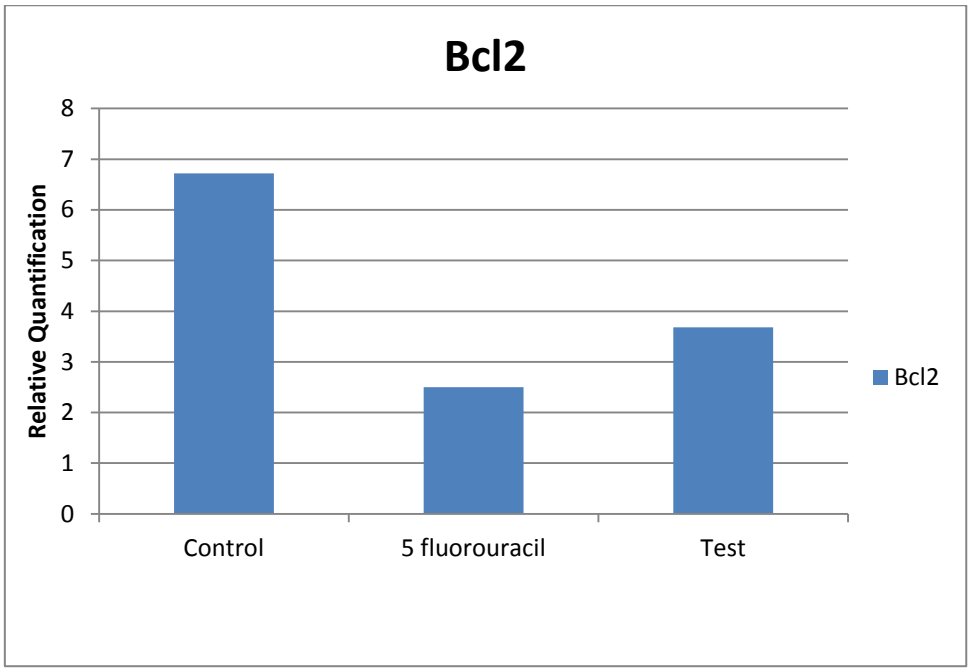


Figure 12: Graphical representation of Bcl-2 gene expression

6. DISCUSSION

Cancer is considered as a serious health problem worldwide. Cervical cancer is the third most common cancer in women. It is one of the leading cause of cancer mortalities in most of the countries in the world with approximately 2,88,000 deaths every year.

Natural phytochemicals derived from medicinal plants have attained a greater significance in potential management of several diseases including cancer. Several researches have been carried out in evaluation of plant extracts as prophylactic agents which offer greater potential to inhibit carcinogenic process.[53,54]

The mechanism of inhibition of tumour progression by natural phytochemicals range from inhibition of genotoxic effects, increased anti inflammatory and anti oxidant effect, inhibition of cell proliferation, protection of intracellular communications to modulate apoptosis and signal transduction pathways.

Amomum subulatum or larger cardamom is a perennial bush of the ginger family.[55]

The essential oil, obtained from the steam distillation of the crushed seeds of Larger Cardamom, contain cineole. Cineole gives the herb its aroma as well as its digestive and appetizer properties. Its antioxidant properties havealso been reported. It has been reported to have anti diabetic, anti fungal, anti oxidant, anti microbial, anti-inflammatory activity. There was no previous study to prove its anti cancer activity with special focus to apoptosis and gene expression study in spite of presence of flavonoid and triterpenoid (1,8 cineole) which is considered as an agent responsible for anti cancer activity. So the study was carried out to evaluate the anticancer effect of ethanolic extract

of fruit of *Amomum subulatum* and the gene expression levels to determine its role in cancer pathology using *in vitro* methods.

Dried fruits of *Amomum subulatum* were powdered and extracted with solvents like acetone, ethanol and water. Phytochemical analysis of acetone, ethanol and aqueous extract of *Amomum subulatum* showed the presence of proteins, carbohydrates, flavanoids, terpenoids, steroids, phenols and tannins. Cytotoxic activity was carried out in cervical cancer cell line HeLa with ethanolic extract and was compared with standard drug 5 Fluorouracil. Test for cytotoxicity was carried out by MTT assay and IC50 value of ethanolic extract was found to be 62.5 µg/ml. IC50 value of 5 Fluorouracil was found to be 7.8 µg/ml. Linearity was expressed with the help of graph plotted in Microsoft excel.[55]

Apoptotic study was carried out by Microscopic analysis and DNA fragmentation. HeLa cells after incubation with IC50 concentration of ethanolic extract for 48 hours were subjected to microscopic studies. On light microscopic observation of ethanolic extract of *Amomum subulatum* treated HeLa cells, typical morphological features of apoptosis like destruction of monolayer, reduction of HeLa cell population, reduction of cell volume, loss of integrity of membrane which resulted in crooked and vesicle shape of the membrane and chromatin condensation were observed when compared to cells without any treatment.

Apoptotic effect of IC50 concentration of ethanolic extract of *Amomum subulatum* treated HeLa cells were further confirmed with the help of fluorescence microscopy using acridine orange and ethidium bromide. Acridine orange is a vital dye capable of staining both dead and live cells, where as ethidium bromide will stain only cells that have lost their membrane integrity. On examination of cells without any

treatment under fluorescent microscope, the cells were stained green in colour representing viable or live cells, whereas examination of cells after treatment with ethanolic extract showed reddish or orange colour with loss of membrane integrity and leakage of cytoplasmic contents representing dead cells and the obtained results were similar to those reported by Shahrul Hisham Zainal Ariffin et al[50] in Hep G2 cells. This led to confirmation that ethanolic extract of *Amomum subulatum* showed apoptotic effect in cervical cancer cell line HeLa.

DNA fragmentation study was carried out by extracting DNA from the cells after treatment with IC50 concentration of ethanolic extract of *Amomum subulatum* and standard 5 Fluorouracil for 48 hours and also from cells without any treatment. DNA agarose gel electrophoresis showed cleavage of chromosomal DNA into oligonucleosomal fragments in HeLa cells treated Ethanolic extract of *Amomum subulatum* and 5 fluorouracil, there was no fragmentation seen in control. This shows that Ethanolic extract of *Amomum subulatum* has anticancer activity. The results obtained were similar to the results shown by Abhimanyu kumar Jha et al[51] using SiHa cell line. This fragmentation of DNA indicated the characteristics of apoptotic cells. Thus ethanolic extract of *Amomum subulatum* causes DNA damage in HeLa cells, thereby inducing apoptosis.

Cancer DNA markers like p53, Bcl2, plays a major role in cancer pathology and their expression levels determine the progression of the disease. The expression levels of p53 and Bcl2 were studied in cells treated with IC50 concentration of ethanolic extract of *Amomum subulatum* and 5 Fluorouracil by RT-PCR methodology using SYBR green.

The expression levels of p53 was found to be increased in cells treated with ethanolic extract of *Amomum subulatum* and in cells treated with 5 Fluorouracil when

compared to cells without any treatment, indicating the ability of ethanolic extract to up regulate p53 and promote apoptosis. The results obtained was similar to those obtained by Azizi et al.[49]

Bcl-2 and its family of proteins play a significant role in regulation of Apoptosis. Bcl-2 plays a major role in cancer and its resistance thereby interfering with therapeutic action of chemotherapeutic drugs. High expression of anti-apoptotic members like Bcl-2 found in human cancers leads to neoplastic cell expansion by interfering with normal cell death mechanism. Decrease in expression of Bcl-2 leads to apoptosis. In this study, the expression levels of Bcl-2 in ethanolic extract treated cells and 5 Fluorouracil treated cells was found to be decreased when compared to expression in cells without any treatment which implies that apoptosis in HeLa cancerous cells may be due to decreased expression of Bcl-2. The results obtained were similar to those reported by Gul Ozcan Arican et al[39] in Hela cells.

7. CONCLUSION

- Phytochemical analysis of acetone, ethanol and aqueous extracts of *Amomum subulatum* revealed the presence of proteins, terpenoids, tannins, steroids, phenols and flavonoids.

- In this study, ethanolic extract of *Amomum subulatum* showed cytotoxic activity in cervical cancer cell line HeLa by MTT assay.

- The Apoptotic effect was confirmed by loss of membrane integrity, chromatin condensation, leakage of cytoplasmic contents and fragmentation of DNA by microscopic methods.

- Its apoptotic and anti cancer effect may be due to up regulation of genes like p53 and down regulation of gene Bcl-2, which was confirmed by RT-PCR.

- The ethanolic extract of *Amomum subulatum* possess anti cancer effect and for future perspective, it can be further confirmed by isolating the compounds responsible for the activity and studying the exact mechanism by which the plant possess this activity and confirm the results using *in vivo* animal models.

8. LIST OF ABBREVATIONS

5-FU	5-Fluorouracil
ALL	Acute Lymphocytic Leukemia
Bax	Bcl-2 associated X protein
Bcl-2	B-cell lymphoma 2
BMI	Body Mass Index
bp	base pairs
BRCA1	Breast cancer susceptibility protein
cDNA	Complementary DNA
CIN	Cervical intraepithelial neoplasia
CLL	Chronic Lymphocytic Leukemia
DBD	DNA Binding Domain
DMSO	Dimethyl sulfoxide
DNA	Deoxyribo Nucleic Acid
dNTP	Deoxynucleotide Triphosphate
DTT	Dithiothreitol
ELISA	Enzyme-linked immunosorbent assay
FBS	Foetal bovine serum
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
HeLa	Henrietta Lacks
Hgb	Hemoglobin
HPV	Human Papilloma Virus
IC50	Median inhibition concentration
IFN-γ	Interferon Gamma
IL	Interleukin
INCTR	International network for cancer treatment and research
JNK	c-Jun N-terminal kinases
KDa	kilo Dalton
LED	Light-emitting diode
MAPK	Mitogen-Activated protein kinases
MCF-7	Michigan Cancer Foundation-7
Mdm2	Mouse double minute 2 homolog

MEM	Minimum Essential Medium
CHOP	Chemotherapy regimen used in the treatment of non-Hodgkin lymphoma
MTT	Dimethyl thiazolyl diphenyl tetrazolium salt
MuLV	Murine Leukemia virus
NaOH	Sodium Hydroxide
NF-κB	Nuclear Factor Kappa-Light-Chain-Enhancer Of Activated B cells
NK	Natural Killer cells
OD	Optical density
p53	Protein 53
PBS	Phosphate Buffer Saline
PCR	Polymerase chain reaction
qPCR	quantitative or real time Polymerase chain reaction
RBC	Red Blood Cells
RNA	Ribo Nucleic Acid
rpm	revolutions per minute
RT-PCR	Reverse transcriptase Polymerase chain reaction
SDS	sodium dodecyl sulphate
SiRNA	Small interfering RNA
SYBR	Synergy Brands, Inc.
TAE	Tris-acetate EDTA
Th1	T helper cells 1
TLC	Thin layer chromatography
TNF	Tumor Necrosis Factor
TNFR	Tumor Necrosis Factor Receptor
TP53	Tumor Protein 53
TPVG	Trypsin, PBS, Versene/EDTA, Glucose
VEGF	Vascular endothelial growth factor
WBC	White Blood Cells
WHO	World Health Organisation

9. BIBILOGRAPHY

1. Introduction to cancer: www.nidirect.gov.uk/cancer-an-introduction.

2. **Anand P,Kunnumakkara AB, Kunnumakara AB, Sundaram C, Harikumar KB, Tharakan ST, Lai OS, Sung B, Aggarwal BB** (September 2008)."Cancer is a preventable disease that requires major lifestyle changes".*Pharm. Res.* **25** (9): 2097–116. doi:10.1007/s11095-008-9661-9.PMC 2515569. PMID 18626751.

3. **Ferlay J, Shin HR, Bray F, et al**. GLOBOCAN 2008 v1.2, Cancer Incidence and Mortality Worldwide: IARC CancerBase No.10 [Internet]. Lyon, France: International Agency for Research on Cancer, 2010. Available from http://globocan.iarc.fr.

4. WHO/ICO Information Centre on HPV and Cervical Cancer (HPV Information Centre). Summary report on HPV and cervical cancer statistics in India 2007. [Last Assessed on 2008 May 1]. Available from: http://www.who.int/hpvcentre

5. **Introduction to medical herbalism** http://www.friedli.com/herbs/intro.html

6. **Rahbari R, Sheahan T, Modes V, Collier P, Macfarlane C, Badge RM** (2009). A novel L1 retrotransposon marker for HeLa cell line identification"*BioTechniques* **46** (4): 277–84.

7. **Harsh Mohan.** Textbook of Pathology. Ed 6. New Delhi: Jaypee Publishers; 2010. Page 192 – 203.

8. **Varricchio, Claudette G.** A Cancer source book for nurses. Boston: Jones and Bartlett Publishers. Page. 229; 2004.

9. How many different types of cancer are there?: Cancer Research UK : Cancer Help UK. Retrieved 11 May 2012, www.wikipedia.org.

10. **Anand P, Kunnumakkara AB, Kunnumakara AB, Sundaram C, Harikumar KB, Tharakan ST, Lai OS, Sung B, Aggarwal BB**. Cancer is a preventable disease that requires major lifestyle changes. *Pharm. Res.* 2008; 25 (9): 2097–116

11. **Sasco AJ, Secretan MB, Straif K**. Tobacco smoking and cancer: a brief review of recent epidemiological evidence. *Lung Cancer.* 2004; 45(2): 3–9.

12. Cancer. *World Health Organization*. Retrieved 9 January 2011, www.wikipedia.org.

13. **Longo, Fauci, Kasper, Hauser, Jameson, Loscalzo.** Harrison's Principles of Internal Medicine. Ed 18. Mc Graw Hill; 2012. Page 663 – 664.

14. What Causes Cancer of the Cervix? .American Cancer Society. 2006-11-30. Archived from the original on 2007-10-13. Retrieved 2007-12-02.

15. **Kumar V, Abbas AK, Fausto N, Mitchell RN** (2007). *Robbins Basic Pathology* ((8th ed.) ed.). Saunders Elsevier. pp. 718–721. ISBN 978-1-4160-2973-1.

16. **Philomena George.** P53 how crucial is its role in Cancer? *Int J Curr Pharm Res.* 2011; 3: 19- 25.

17. **Ziwei huang.** Bcl-2 family proteins as targets for anticancer drug design. *Oncogene.* 2000; 19: 6627-6631.

18. **Alberts et al.** *Molecular Biology of the Cell;* 5th Edition, Garland Science, 2008 Chapter 20; Cancer, pp.1230-1256

19. **Kumar V, Abbas A, Fausto N**: *Robbins and Cotran Pathologic Basis of Disease* 7th ed, Elsevier/ Saunders, 2005; Chapter 7: Oncogenes and Tumor Suppressor Genes pp. 292-306.

20. **Woodman C et al.,** The natural history of Cervical HPV infection: unresolved issues. Nat Rev Cancer 2007. 7:11.

21. **Zur Hausen H.** Papillomaviruses and cancer: from basic studies to clinical application. Nat Rev Cancer 2002. 2:342.

22. **Canavan TP, Doshi NR** (2000). Cervical cancer *Am Fam Physician* **61** (5): 1369–76.PMID 10735343.

23. The American Joint Committee on Cancer (AJCC). Staging cervical cancer – 7th edition. 2010. UpToDate. www.uptodate.com (accessed 2011).

24. De Los Santos J et al. Invasive cervical cancer: management of early stage disease (FIGO 1A, 1B1, nonbulky 2A1) and special circumstances. 2011. UpToDate. www.uptodate.com (accessed January 2012).

25. De Los Santos J et al. Management of stages 1B2, bulky 2A2, and locally advanced disease. 2011. UpToDate. www.uptodate.com (accessed January 2012).

26. High-dose rate brachytherapy for carcinoma of the cervix. 2006. National Institute for Health and Clinical Excellence (NICE).

27. Plante M. Patient information: fertility preservation in women with early stage cervical cancer. 2011. UpToDate. www.uptodate.com (accessed January 2012).

28. **Kumar gopal et al.,** *Amomum subulatum* Roxb: an overview in all aspects. International research journal of pharmacy 2012; 3(7): 96-99.

29. Anonymous. The Ayurvedic pharmacopoeia of India. Government of India. 1999; 1(2): 158-159.

30. **Patel amit et al.,** Anti-diabetic activity of *Amomum subulatum* Roxb. Fruit constituents. IJPI 2012.

31. **Mihir parmar, purvi shah, vaishali Thakkar, Tejal R. Gandhi.,** Hepatoprotective activity of *Amomum subulatum* Roxb against ethanol induced-induced liver damage. International journal of Green pharmacy 2009: 250-254.

32. HeLa cell line: cervical adenocarcinoma cell line – General information http://HeLa transfection.com/.

33. **Sheng-hua zhang et al.,** Morphological study on apoptosis HeLa cells induced by soyasaponins. Toxicology invitro 21 2007; 820-826.

34. **Aied M Alabsi et al.,** Apoptosis Induction, Cell Cycle Arrest and in Vitro Anticancer Activity of Gonothalamin in a Cancer Cell Lines. Asian Pacific J Cancer Prev 2012; 13 (10): 5131-5136.

35. **SANJAY PATEL et al.,** In-vitro cytotoxicity activity of *Solanum nigram* extract against HeLa cell line and vero cell line . International journal of pharmacy and pharmaceutical sciences 2009; 38-46.

36. **Ali alshehri, Hafez.E.Elsayed.** Molecular and bio chemical evaluation of anti proliferative effect of Cinchorium endivia, L phenolic extracts on breast cancer cell line MCF-7. *Journal of Biotechnology and pharmaceutical Research.* 2012 June; 3(4): 74-82.

37. **Kameshwaran, V.Suresh, G.Arunachalam, S.K.Kanthlal, M.Mohanraj.** In vitro and In vivo anticancer activity of methanolic extract of Tecoma stans flowers. *IRJP*. 2012; 3 (3): 246 – 251.

38. **Anjana munshi et al.,** Induction of apoptosis in HeLa cells by chloroform fraction seed extracts of *nigella sativa.* Cancer cell International 2009; 1-8.

39. **Gul ozcan arican, Ozgur cakir, Ercan arican, Neslihan turgut kara, Ozlem dagdeviren, Sule ari.** Effect of Geven root extract on proliferation of He La cells and Bcl-2 gene expressions. *African Journal of Biotechnology.* 2012 ; 11(18) : 4296 – 4304.

40. **Shruti Nair, KN Varalakshmi.** Anticancer, cytotoxic potential of *Moringa oleifera* extracts on HeLa cell line. *Journal of natural pharmaceuticals.*2011; 2(3): 138 – 142.

41. http://www.nature.com/cr/journal/v10/n3/full/7290049a.html

42. **Nagata.S, Enari.M, Sakahira.H, Yokoyama.H, Okawa.K, Iwamatsu.A.** A caspase activated DNase that degrades DNA during apoptosis, and its inhibitor ICAD. *Nature.* 1998; 391(6662): 43-50.

43. **Kanimozhi D et al.,** In-Vitro Anticancer Activity of Ethanolic Extract of Cynodon dactylon Against HEP-2, HELA and MCF-7 Cell Lines. International Journal of Scientific Research and Reviews 2012; 1(1): 10-23.

44. **Ali Alshehri.** Molecular and Biochemical evaluation of Antiproliferative effect of Cichorium endivia L. phenolic extracts on cancer cell line HCT-116. Academic journal of Cancer Research. 2012; 5(2): 53-60.

45. **Ali A Alshatwi, Gowhar Shafi, Tarique N Hasim, Amal A Al- Hazzani, Mohammed A Alsaif, Mohammed A Alfawaz, Ky Lei, Anjana munshi.** Apoptosis mediated inhibition of Human breast cancer cell proliferation by lemon citrus extract. *Asian pacific journal of Cancer prevention.* 2011; 12: 1555-1559.

46. **Wallis TE,** Textbook of Pharmacognosy. New Delhi: CBS Publishers and distributors; 1985. Page 133 – 248.

47. http://ww.atcc.org

48. **S.K.Gupta.** Drug Screening Methods (Pre clinical Evaluation of New Drugs), 2nd Edition. Jaypee Brothers Medical Publishers. 2009; Page No. 168 – 169.

49. **Azizi E, Abdolmohammadi M.H, Fouladdel Sh, Shafiee A, Amin Gh, Ghaffari S.M.** Evaluation of p53 and Bcl-2 genes and proteins expression in human breast cancer T47D cells treated with extracts of Astrodaucus persicus(Boiss.) Drude in comparison to tamoxifen. *Daru.* 2009; 17(3): 181 – 186.

50. **Shahrul Hisham Zainal Ariffin, Wan Haifa Haryani Wan Omar, Zaidah Zainal Ariffin, Muhd Fauzi Safian, Sahidan Senafi, Rohaya Megat Abdul Wahab.** Intrinsic anticarcinogenic effects of Piper sarmentosum ethanolic extract on a human hepatoma cell line. *Cancer Cell Int.* 2009: 9(6).

51. **Abhimanyu kumar Jha, Meenakshi Jha, Jagdeep kaur.** Ethanolic extracts of Ocimum sanctum, Azadirachta indica and Withania somnifera causes apoptosis in SiHa cells. *Research journal of Pharmaceutical, Biological and Chemical sciences.* 2012; 3(2): 557- 562.

52. Molecular and Biochemical evaluation of Antiproliferative effect of Cichorium endivia L. phenolic extracts on cancer cell line HCT-116. Academic journal of Cancer Research. 2012; 5(2): 53-60.

53. **Mehta RG, Murillo G, Naithani R, Peng X.** Cancer chemoprevention by natural products: how far have we come? *Pharm Res.* 2010; 27: 950-61.

54. **Desai AG, Qazi GN, Ganju RK, et al.** Medicinal plants and cancer prevention. *Curr drug metab.* 2008; 9: 581-591.

55. **Rajesh M. Patel, Sahil K. Patel .** Cytotoxic activity of methanolic extract of Artocarpus heterophyllus against A549, Hela and MCF-7 cell lines. *Journal of Applied Pharmaceutical Sciences* . 2011; 1(7): 167 – 171.